Liquid Chromatography-Mass Spectrometry, an evolution in doping analysis

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Promotor: Prof. Dr. F.T. Delbeke

Dankwoord

Het doctoraat is geschreven, dus…tijd om enkele mensen te bedanken!

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List of Abbreviations

3OHSTAN	3'hydroxystanozolol
6α-OH-AD	androst-4-ene-6α-ol-3,17-dione
6α-OH-T	androst-4-ene- 6α, 17β-diol-3-one
6β-OH-AD	androst-4-ene-6β-ol-3,17-dione
6β-OH-T	androst-4-ene- 6β,17β-diol-3-one
6-oxo-AD	androst-4-ene-3,6,17-trione
16OHSTAN	16β-hydroxystanozolol
17TREN	17α–trenbolone
ACN	acetonitrile
ACTH	adrenocorticotropic hormone
AD	androst-4-ene-3,17 dione
ADHD	attention deficit hyperactivity disorder
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
APLI	atmospheric pressure lazer ionisation
APPI	atmospheric pressure photo ionisation
Da	dalton
DoCoLab	Doping Control Laboratory
ELISA	enzyme linked immuno selective sorbent assay
EPO	erythropoetin
ESI	electrospray ionisation
GC	gas chromatography
GC-MS	gas chromatography – mass spectrometry
GH	growth hormone
hCG	human chorionic gonadotrophin
IS	internal standard
GES	gestrinone
HAc	acetic acid
HCOOH	formic acid

HCl	hydrochloric acid
HPLC	high performance liquid chromatography
ISO	International Standard Organization
IU	international units
MS	mass spectrometry
IOC	International Olympic Committee
LC	liquid chromatography
LC-MS	liquid chromatography- mass spectrometry
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantitation
m/z	mass- to-charge ratio
MS^n	multiple mass spectrometry with n>1
MS/MS	tandem mass spectrometry
MBHFB	N-methyl-bis-heptafluorobutyramide
MBTFA	N-methyl-bistrifluoroacetamide
MDA	3,4-methylenedioxyamphetamine
MDEA	3,4-methylenedioxyethylamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MeOH	methanol
MRPL	minimum required performance limit
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
NPD	nitrogen-phosporus selective detection
NSAIDs	non steroidal anti-inflammatory drugs
OFN	oxygen free nitrogen
PCR	polymerase chain reaction
RP	reversed-phase
rpm	revolutions per minute
RRT	relative retention time
RT	retention time
RSD	relative standard deviation
SD	standard deviation

S/N	signal-to-noise ratio
T	testosterone
TFAA	trifluoroacetamide
THC	tetrahydrocannabinol
TIC	total ion chromatogram
TOF	time of flight
TUE	therapeutic use exemption
TSP	Thermo separation products
THG	tetrahydrogestrinone
UV	ultra violet
WAADS	World Association of Anti-Doping Scientists
WADA	World Anti-Doping Agency
XTC	MDMA

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Aim of this study

The aim of the work was to improve the screening of several classes of doping agents by using LC-$MS^{(n)}$.

The drive to compete and win is as old as mankind. Hence, athletes have tried to achieve an unfair advantage over fellow competitors by the use of doping. To guarantee fairness amongst competitors and towards the public and to protect the health of the athletes, doping controls are organised by the anti-doping organisations.

Doping laboratories play a key role in the analysis of the samples. Therefore they try to have the newest analytical techniques at their disposal. During the 1960s gas chromatography-mass spectrometry was introduced as a new analytical technique. Because GC-MS was the only available chromatographic-mass spectrometric technique for more than 30 years, it was used for the unequivocal identification of doping substances. One of the requirements for GC-MS analysis is that the analytes are volatile, which often requires their derivatisation. Unfortunately some non-volatile compounds cannot be derivatised. Moreover, some derivatives are thermally instable and are degraded in the injector. In particular for diuretics, corticosteroids and beta-blockers derivatisation was poor. Hence these compounds were screened for by other techniques including HPLC-UV or ELISA.

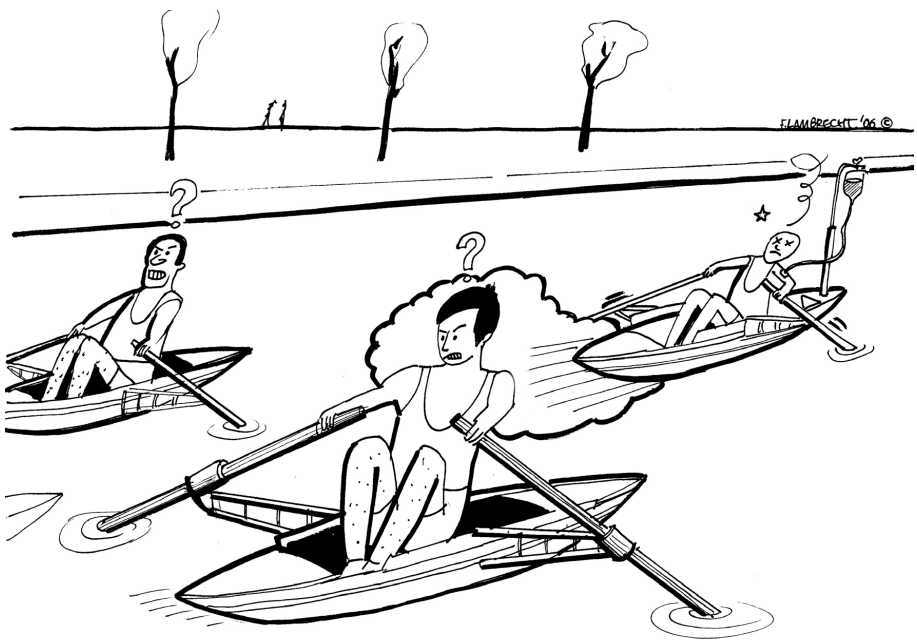
Separation of compounds by liquid chromatography does not require a derivatisation step and in the 1980s much attention was paid to couple this separation technique with mass spectrometry. At the beginning of the 1990s commercial instrumentation became available and researchers focused primarily on substances for which GC-MS detection was difficult or impossible. In 1991, an early approach to the analysis of diuretics by LC-MS showed promising results. Despite the good results, technical limitations impeded its routine application. In the next years the quality of LC-MS instrumentation improved with a decrease of the purchase costs.

In 1998 an LCQ-Classic (Thermo, San José, California, USA) was purchased by the Department of Pharmacology, pharmacy and toxicology. Pilot investigations on this instrument for diuretics, corticosteroids and beta-blockers showed that LC-MS allows to detect these compounds in urinary matrices with a minimum of sample preparation in a sensitive and specific way.

Finally, in 2001 the first LC-MS instrument (LCQ-Deca, Thermo, San José, California, USA) entered the Doping Control Laboratory with the financial support of the National Lottery. Initially, no one could expect that this single LC-MS instrument would be the start of a full LC-MS laboratory unit where currently three LC-MS systems are working routinely day and night.

The research described in this work was performed within the framework of a project funded by the Flemish Community to develop fast, accurate and flexible LC-$MS^{(n)}$ methods for the routine detection of the previously pilot-investigated diuretics, corticosteroids and beta-blockers in urine. Anabolic steroids were added to the project when THG, a designer anabolic steroid, was discovered. This compound could not be detected by the routine GC-MS screening method and the detection by LC-MS was mandatory. Due to the experience with the detection of stimulants in nutritional supplements, their detection in urine was also investigated.

Chapter I: Introduction



1. Doping: history, current status and future

Throughout history, athletes have sought foods and potions to enhance physical performance.

The Roman gladiators of Circus Maximus used stimulants mixed with alcohol to overcome fatigue and injuries and Scandinavian warriors (The Berserkers) ate hallucinogenic mushrooms to gear up for battle [1].

The first competitive athletes charged for doping were swimmers in Amsterdam in the 1860s [2]. In the late 19th century European cyclists were using substances like caffeine and ether-coated sugar cubes to reduce pain and delay fatigue. Unfortunately, it took another 40 years before the first doping test was introduced [3]. These tests were primitive compared to current techniques. Hence, athletes were not afraid to get caught and doping (ab)use was widespread.

Shortly after the Second World War, amphetamine type stimulants became very popular [3] resulting in several lethal cases. One of the most well-known doping victims in that period was Tom Simpson who died in 1967 on the Mont Ventoux from a combination of exhaustion, alcohol and amphetamines. The year after his death athletes were tested for the first time at the Olympic Games in Mexico City, 1968 [4]. Although these first tests were mainly focusing on stimulants, the detection of other compounds was necessary as well. Advances in organic chemistry in the 1950s and 1960s yielded a wide variety of pharmacologically active compounds including diuretics, beta-blockers, corticosteroids and anabolic steroids. These compounds were intensively used by athletes during the 1970s.

By the end of the 1970s and especially during the 1980s, biotechnology successfully advanced. PCR and genetical modification allowed for the in-vitro production of huge amounts of protein based medicines including GH, insulin and derivatives, ACTH and erythropoetin known as EPO. These protein based products were helpful in the treatment of many diseases, unfortunately they found their way into doping as well.

While the detection of GH is still under investigation, reliable tests for the detection of EPO [5] are available since the Sydney Olympic Games in 2000.

Because of successes in the battle against doping resulted in an increased risk of getting caught, athletes tried "older" forms of doping. At the Vuelta in 2004, a cyclist used blood transfusion.

Another way to avoid getting caught is the use of "designer" steroids. Designer steroids are chemically modified anabolic steroids which were developed in the 1960s and 1970s. These substances were undetectable by the doping laboratories until 2003 when the designer steroid THG was found after an anonymous tip [6].

In the past few years, experiments with gene therapy to treat diseases were investigated and researchers have successfully beefed up muscles of mice and baboons by genetic modifications. However the gene therapy could be applied to strengthen specific muscles in an athlete. Although no (doping) applications are available yet, WADA included genetic doping on the list of prohibited methods since January 1st, 2003 [7].

Which new doping substances the future will bring is difficult to predict. The abuse of new doping substances or methods only reach doping authorities by rumours or anonymous tips. Nevertheless doping authorities are carefully watching scientific advances which may be abused in the future.

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2. Liquid chromatography-mass spectrometry

Chromatographic separation

The evolution of chromatography started in 1906 when Swett mentioned for the first time the word chromatography [1]. Chromatography is a physical separation method in which the components are selectively distributed between two immiscible phases: a mobile phase flowing through a stationary phased bed. The following types of LC techniques can be distinguished: adsorption (normal-phase and reversed-phase), ion-exchange and size- exclusion chromatography. Nowadays reversed-phase chromatography is the most commonly used separation technique. The reason is the broad application range; reversed-phase chromatography is able to handle compounds of a diverse polarity and molecular mass. The retention of an analyte depends on its partition between the polar mobile phase and the non-polar stationary phase. Reversed phase columns consist of a silica or polymeric carrier and a coating of long chain saturated hydrocarbons or other non-polar functional groups. The most popular packing material is octadecylsilane with an 18-carbon aliphatic chain. The covalent bonds between the silica carrier and the aliphatic chain are thermostable and chemically stable, generally in a pH range between 2 and 8. Solvents most often used are water, methanol and acetonitrile. The partitioning of an analyte between the mobile and stationary phase depends upon hydrophobic interactions between the sample and the mobile phase. Small polar molecules elute more rapidly than large apolar ones [2,3].

Reversed phase high pressure liquid chromatography RP-HPLC was popularised throughout the 1970s as a sophisticated improvement for open columns and provided more precise and rapid separations required in many areas including doping analysis. Initially, HPLC with UV detection was merely used for confirmation of suspected urine samples by comparing retention time and UV-spectrum with reference standards. Routine HPLC-UV screening methods were introduced in the 1980s for the detection of diuretics, xanthines and NSAIDs [4-6]. Unfortunately, this detection technique has only limited sensitivity, selectivity and specificity. At the beginning of the 1990s mass

spectrometry was introduced as a new sensitive detection technique for HPLC in the field of doping analysis [7].

When HPLC is coupled to MS, some considerations are mandatory in the selection of the solvents for the mobile phase. The commonly used solvents in reversed-phase chromatography (water, methanol and acetonitrile) are also ideal for LC-MS. Unfortunately, non-volatile solvent additives which are frequently used for LC separations, are not compatible for LC-MS analysis; phosphate buffers are included in this category. These solvents can crystallize in the ion source and prevent the mass spectrometer from functioning properly. Instead of these non-volatile buffers, volatile buffers, such as ammonium acetate and formate, can be used.

Interfacing techniques and ion suppression

Interfacing LC with MS is not straightforward since the analytes leaving the LC column (flow: 0.1-1 mL/min) are dissolved in the effluent at atmospheric pressure, while the MS is constructed to detect ions in the gas phase under high vacuum. In LC-MS the effluent must be evaporated and pumped away prior to the introduction of the ionised analytes in the mass spectrometer.
One of the first attempts to simultaneously remove these high amounts of liquid and to generate ions was reported by Tal'rose et al., using a capillary inlet interface between the LC and MS [8]. Afterwards other types of interfaces have been developed including thermospray and fast atom bombardment [9].
The breakthrough in LC-MS however was achieved with the development of two techniques for API, i.e. electrospray ionisation (ESI) [10] and atmospheric pressure chemical ionisation (APCI) [11].

Electrospray ionisation (ESI)

ESI is an ionisation technique typically used to analyse polar compounds.
Many samples that previously were not suitable for mass spectrometric analysis (e.g. heat-labile compounds or high molecular weight compounds) can be analysed by ESI.

A prerequisite for ESI is that the compound of interest generates a preformed ion in solution. Acidic molecules form negative ions $[M-H]^-$ in solutions with high pH, and basic molecules form positive ions $[M+H]^+$ at low pH. The term *preformed ion* includes adduct ions as well. ESI can be used in either positive or negative ion polarity mode. A positively charged ESI needle detects positive ions and a negatively charged needle is used for negative ions.

Small molecules generally produce mass spectra consisting of a single charged ion. Large molecules (peptides and proteins) typically produce mass spectra consisting of multiple charged ions. The resulting (complex) mass spectrum can be mathematically manipulated to determine the molecular weight of the substance.

Three steps are involved in the formation of the gas-phase ions. First, the solution containing the analyte, eluting from the analytical column, passes through a needle (the electrospray needle) that has a high potential applied to it (typically in the range from 2.5 to 5 kV). This forces the spraying of charged droplets from the needle with a surface charge of the same polarity to the charge on the needle (Figure 1, A).

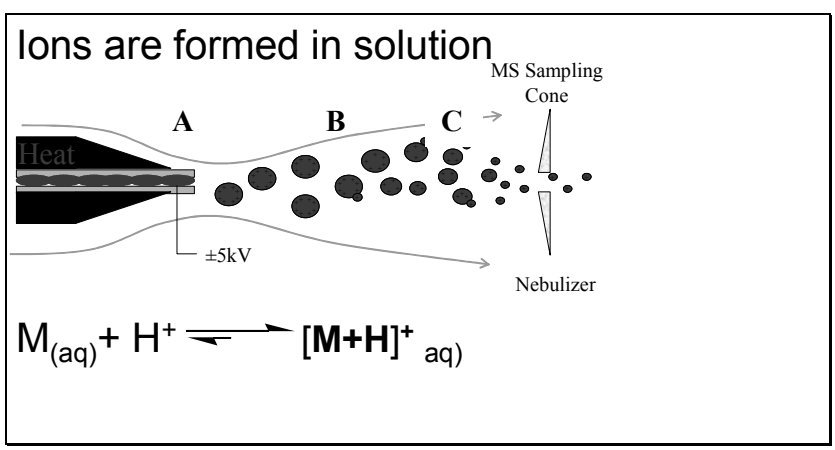

Figure 1: Schematic view of the ESI-ionisation process.

Subsequently, the droplets are repelled from the needle towards the source sampling cone on the counter electrode. As the droplets traverse the space between the needle tip and the cone, solvent evaporation occurs and the droplets shrink (Figure 1, B) until reaching the point that the surface tension can no longer sustain the charge and a

coulombic explosion occurs and the droplets are ripped apart. This produces smaller droplets. The process (further reduction of droplet size) is repeated and naked charged analyte molecules (Figure1, C) are generated. These charged analyte molecules can be single or multiple charged [12].

Atmospheric pressure chemical ionisation (APCI)

Like ESI, APCI is also a soft ionisation technique. APCI provides molecular weight information for compounds of medium polarity. APCI differs from ESI in the way the ionisation occurs. First the analyte solution is introduced into a heater (Figure 2, A) where complete desolvation occurs (operating temperatures between 350 and 550 °C). Then the gas phase analytes leave the heating coil and interact with protonated or deprotonated solvent molecules (Figure 2, B). These protonated or deprotonated solvent molecules result from a plasma generated by a corona discharge needle (Figure 2) with a fixed discharge current (typically in the range from 3 to 8 μA). Due to the interaction of the protonated or deprotonated solvent molecules with the analytes the ionisation occurs. APCI is typically used to analyse molecules with molecular weights up to about 2000 Da [12].

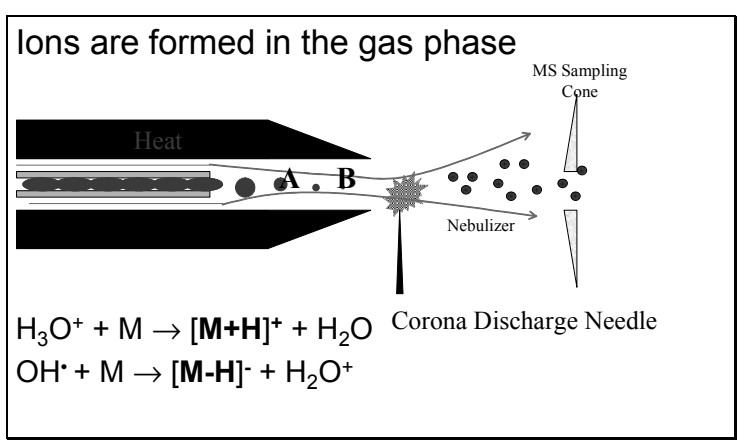

Figure 2: Schematic view of the APCI-ionisation process.

Ion suppression

Ion suppression appears as a kind of matrix effect specifically linked to LC-MS. It occurs during the evaporation step and is observed with both ESI and APCI. APCI however is considered to be less susceptible to this phenomena [13].
The main problem source commonly reported is the presence of endogenous substances, i.e. organic or inorganic molecules present in the sample that are retrieved in the final extract. Among this first group of ion suppressor agents, ionic species (inorganic electrolytes and salts), highly polar compounds and various organic molecules including carbohydrates, amines, urea, lipids, peptides, analogous compounds or metabolites with a chemical structure close to the target analyte one [14,15].
In order to overcome this problem an internal standard eluting near the analyte can be used in order to compensate for the suppression. The best way to achieve this goal is the use of deuterated or ^{13}C-labelled analogues. These deuterated compounds however, are not always available. Another approach is to force the IS to elute together with the analyte of interest or in case of multiple analytes inbetween the quantified substances [14].

Mass spectrometry

After ionisation in the interface the analytes are transferred into the mass spectrometer where the actual detection is performed.
Mass spectrometry is based on the measurement of mass-to-charge (m/z) ratios of ions. All molecular ions are, in principle, accessible by mass spectrometry, making it a universal method for chemical analysis. Its implementation requires suitable methods of ion generation, ion analysis and ion detection.

Several types of mass spectrometers have been described including sector mass analyzers (single focusing and double focusing), quadrupole mass analyzers, ion trap mass analysers, time-of-flight mass analysers and Fourier-transform ion cyclotron resonance mass analysers. Recently a new type of mass spectrometer was introduced, called Orbi-Trap® (Thermo, San José, California, USA). Quadrupole and ion trap mass

spectrometers are the most commonly used. All experiments described in this work were performed on ion trap instruments.

Ion trap principle

The ion trap consists of two identical endcap electrodes, the entrance endcap electrode and exit endcap electrode, as well as the ring electrode (Figure 3).

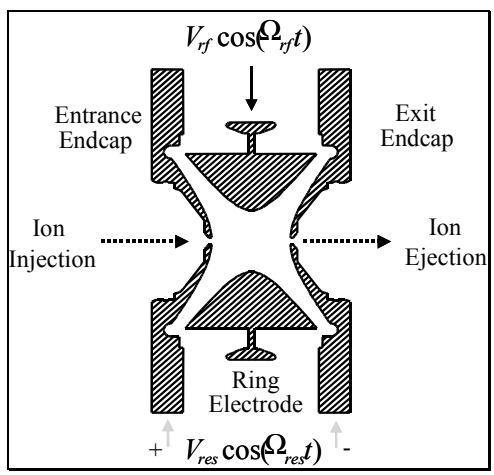

Figure 3: Schematic view of the ion trap.

The electrodes are spaced apart by quartz spacer rings, creating a hollow space in which the ions are trapped. Trapping occurs by applying a three dimensional oscillating electric field between the electrodes. The ions pass in (ion injection) and out (ion ejection) of the traps by holes in the endcaps. In theory, the trapped ions can remain indefinitely in the trap by the oscillating electric field. However, in order to be detected, they need to be scanned out to the detection system (ejection). Therefore the frequency of the DC voltage applied to the ring electrode is ramped making the ions being ejected from the trap to the detector [16].

Ion trap operation

The ion trap mass spectrometer can be operated in different ways:
Full scan MS*:* All the ions are collected and then ejected, resulting in a spectrum that displays the ion count of each mass initially entering the trap.
SIM*:* In this case, the ions are also collected, but during the time in which the ions are maintained in the trap, voltages are altered to isolate a single ion or ion window, thus retaining only the ion of interest and purging everything else. In the ejection step the only ion scanned out is the isolated ion.
MS^2*:* In this situation, the ions are collected and a single ion is isolated as in the SIM experiment. Then voltages are applied to excite and fragment that precursor ion into product ions all of which can then be scanned out. A feature of the ion-trap instrument is that it can provide multiple stages of mass spectrometry (MS^n). A precursor ion can be fragmented over and over [16].

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3. Qualitative validation in doping analysis

Introduction

Substances prohibited by WADA must not be present in tested urines. This is the main criteria underlying doping control. As doping control routinely concern several hundred substances and laboratories report results within 24 h, analytical procedures do not usually include quantification of the screened substances. The length of the list of controlled substances and the time pressures involved in reporting the results are the main differences between doping control and related fields such as the forensic sciences or drug abuse testing, both of which report quantitative results but deal with shorter menus.

Hence, sample analysis in doping analysis can be seen as a two step procedure. A preliminary, fast and comprehensive screening method with a minimum of sample preparation without quantification. For compounds with a threshold (Table 1) [1] a (second) quantitative analysis is performed. For both threshold and non threshold substances a confirmation step is needed for unambiguous confirmation [2].

Table 1: Compounds with a threshold level

Compound	Treshold level
Carboxy-THC	15 ng/ml
Cathine	5 µg/ml
Ephedrine	10 µg/ml
Epitestosterone	200 ng/ml
Methylephedrine	10 µg/ml
Morphine	1 µg/ml
19-norandrosterone	2 ng/ml
Salbutamol	1 µg/ml

Standard methods are generally not available for qualitative doping analysis. Each laboratory develops, validates and document in-house methods for the compounds present on the prohibited list. For example, we confirm hydroxyethylstarch (a plasma volume expander) using LC-MS [3] whether in other doping control laboratories GC-

MS is applied as detection technique [4]. The method for EPO however is standardized and all laboratories use the same analytical procedure [5].

Validation Parameters

LOD, MRPL and Signal-to-noise

To assure that all doping laboratories can report the presence of prohibited substances, their metabolites or markers in a uniform way, a minimum routine detection capability for testing methods has been established called the minimum required performance limit (MRPL) [1] (Table 2).

Table 2: Minimum required performance level for representative substances in the class of prohibited substances and exceptions.

Prohibited Class	Specific Examples/Exceptions	MRPL
Stimulants		0.5 µg/ml
	Strychnine	0.2 µg/ml
Narcotics		0.2 µg/ml
	Buprenorphine	10 ng/ml
Anabolic Agents		10 ng/ml
	Clenbuterol	2 ng/ml
	Methandienone*	2 ng/ml
	Methyltestosterone*	2 ng/ml
	Norandrosterone	1 ng/ml
	Stanozolol*	2 ng/ml
	Epitestosterone	2 ng/ml
Beta-blockers		0.5 µg/ml
Diuretics		0.25 µg/ml
Glucocorticosteroids		30 ng/ml
Peptide Hormones		
	hCG	5 mIU/ml

* As metabolites

The MRPL is not a threshold, nor is it a limit of detection (LOD) or a limit of quantification. Adverse analytical findings may also result from concentrations below the MRPL. WADA states that each laboratory must demonstrate (validate) the ability to achieve the MRPL using representive substances or standards if they are available [6].

Doping laboratories are ISO17025 accreditated [7] but ISO does not specify guidelines for validation and neither does WADA. WADA refers to Eurachem [8] and a decision made by the European Community for residue analysis in animal tissues [9] as validation guidelines for the doping laboratories.

Eurachem describes that it is sufficient to provide an indication of the level at which detection becomes problematic. In other words, the detection limit (LOD) should be determined. They suggest to determine this LOD in 10 independent replicates fortified with standards at different concentration levels. For doping analysis this means fortifying 10 blank urine samples from different persons (randomly chosen) at different levels including the MRPL. The LOD is then determined by establishing the lowest level at which the analyte can be reliably detected. Therefore the signal-to-noise ratio is determined.

Determination of the signal-to-noise ratio is performed by comparing the measured signal with the background signal (Figure 1).

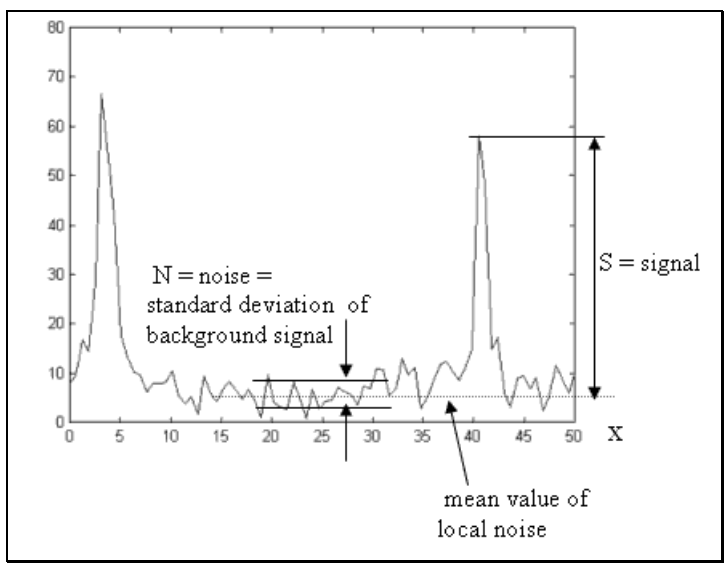

Figure 1: Determination of signal-to-noise ratio (S/N) in chromatography.

A signal-to-noise ratio of 2/1 or 3/1 is generally considered acceptable [10]. In our laboratory a S/N of 3/1 is applied as detection criterium. Ultimately the LOD for a

compound is defined as the lowest fortified level for which in all urine samples the compound of interest can be detected with a S/N of 3.

As stated before, WADA only recommends to prove the ability to achieve the MRPL and the determination of the LOD is not required [6].

Hence another approach for the validation of qualitative methods was developed according to the guidelines for residue analysis in animal tissues [9]. Urine samples have to be fortified at 3 levels (½ MRPL, MRPL, 2x MRPL) and analysed with the method subject to validation. By working in this way sufficient data is obtained to evaluate the method regarding detection capability versus MRPL (Table 3).

Table 3: Interpretation of the validation data obtained after the analysis of fortified samples spiked at 1/2 MRPL, MRPL and 2x MRPL.

	Score*			
½ MRPL	10/10	<10/10	10/10	<10/10
MRPL	10/10	10/10	10/10	<10/10
2x MRPL	10/10	10/10	<10/10	10/10
Conclusion	LOD ≤ ½ MRPL	LOD = MRPL	Adverse finding, validation must be repeated	MRPL not reached, method must be improved

* Score means number of the replicates in which the compound could be identified with a S/N > 3 out of the 10 fortified replicates.

This validation near the MRPL is used when an existing method is slightly modified (minor changes in sample preparation, chromatography or adding an additional SRM transition).

In our laboratory, LOD determination, according to the Eurachem guidelines, is only performed when a new method was developed or for research purposes.

As stated above MRPL's must be evaluated if standards are available. In the case no standards or metabolites are available excretion urine samples are obtained from patients or from excretion studies with healthy volunteers.

Specificity and Selectivity

Definitions of specificity and selectivity are similar in quantitative and qualitative analysis [8]. For specificity it is the ability of a method to determine an analyte of

interest in the presence of other compounds in a sample matrix under the stated conditions of the test. To demonstrate this during validation the blank urine samples used for the fortification are also analysed. No interfering peaks should be present at the retention time of the compound of interest. For the determination of selectivity reference substances from other routine screening methods are analysed.

Repeatability, Reproducibility and Robustness

Accuracy, precision, repeatability and reproducibility are terms which can be easily defined for quantitative validations because numeric results are obtained. Hence for toxicologal and forensic fields, dealing with quantitative assays, conclusive definitions are available [10, 11]. Since qualitative validation results in yes/no binary results (i.e.: detection or no detection) it is difficult to apply these terms. Nevertheless efforts have been made to define repeatability and reproducibility for qualitative screening purposes [9,13].

Repeatability (also termed intra-assay precision) is the closeness of agreement between a series of measurements obtained from different aliquots with identical matrices under same operating conditions and is part of the criteria to evaluate the precision of a method [9]. By analysing 10 samples, fortified at a certain concentration, a statistically sound basis is made to show repeatability of a qualitative method.

Reproducibility and robustness of the methods are not considered during the qualitative validation protocol. Robustness is evaluated through the quality control samples analysed with each batch of samples and reproducibility is evaluated by WADA's proficiency tests [6] and collaborative studies organised by WAADS.

Recovery and ion suppression

Extraction recovery is a measure of the efficiency of the extraction of the analyte from the sample matrix. It is expressed as the ratio of the response obtained when the analyte is submitted to the extraction procedure to that measured when it is determined without the extraction step [9]. Since screening methods are straightforward methods in which different classes of substances can be present recoveries can be poor for some

compounds. The results of the validation guarantees the detection of each compound independently from its recovery. Recoveries are only evaluated for research purposes or to optimize existing screening methods.
Ion suppression in LC-MS can occur when coeluting compounds suppress the ionisation of the analytes [14]. During qualitatitve validation ion suppression is incorporated in the determination of the LOD.
By using 10 different urine samples during the validation process, 10 different urinary matrices are evaluated.

Conclusion

Each laboratory needs to develop, validate, and document in-house methods for the compounds present on the prohibited list.
For screening purposes, the criteria for chromatography as well as mass spectrometry can be different between the doping control laboratories as long as they are scientifically supported by validation data. However, for the unambiguous confirmation the rules are more stringent and have to be compliant with WADA criteria [2].

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Chapter II: Diuretics and Beta-blockers

1. Introduction

Therapeutic use

Compounds discussed in this chapter, diuretics and beta adrenergic blocking agents (i.e. beta-blockers), belong to the group of drugs used in anti-hypertensive therapy. They control high blood pressure in different ways.
Diuretics stimulate the kidneys to produce more urine, flushing excess fluids and minerals from the body resulting in a lowering of the blood pressure. They are also used in the treatment of oedema associated with cardiac or renal insufficiency [1].

Diuretics cover a wide range of chemical products and can be classified according to their chemical structure, their mechanism and primary site of action in the nephron and their diuretic potency. The molecular structures of some selected diuretics and probenecid are shown in Figure 1.

Beta-blockers act directly on the heart by slowing the beating rate. They are also used in the treatment of glaucoma, thyreotoxicosis, anxiety states and tremor [2]. More recently beta-blockers seem to be helpful in the prevention of migraine [3].

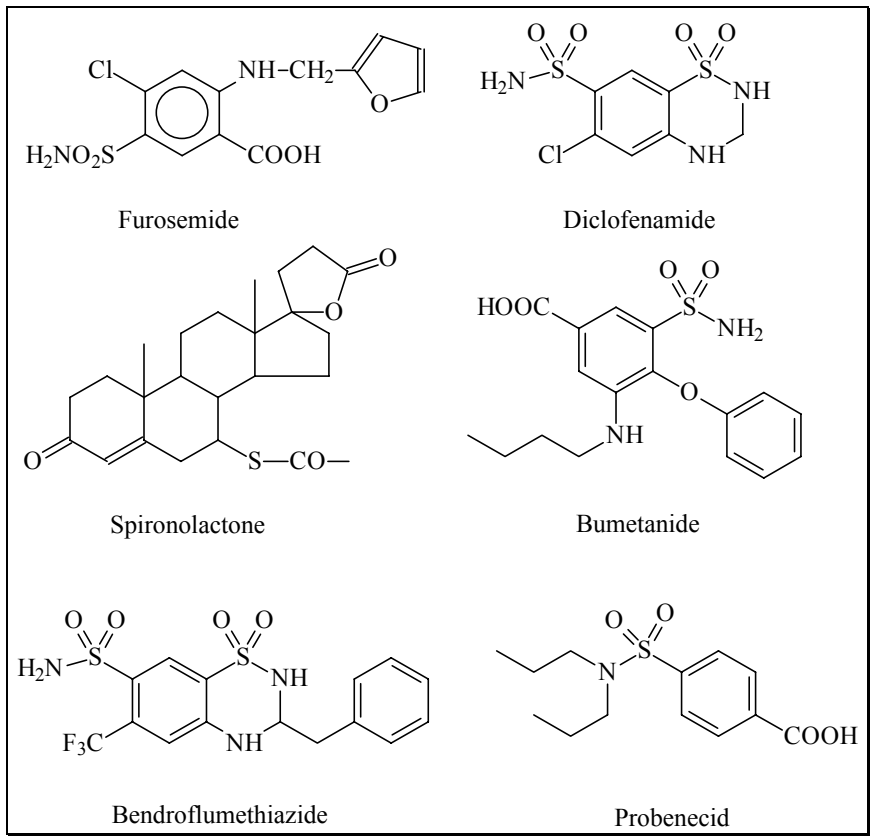

Figure 1: Molecular structures of some selected diuretics and probenecid.

Similarly as for diuretics, beta-blockers cover a wide range of chemical products. Worldwide more than 100 beta-blockers have been synthesised since 1958. One of the first potent beta-blocker commercially available was propranolol (Figure 2). Depending on their site of action beta-blockers can be divided in β_1 and β_2-receptor antagonists [2]. Another way of classifying beta-blockers is according to their chemical structure [4]. The molecular structures of some selected beta-blockers are presented in Figure 2.

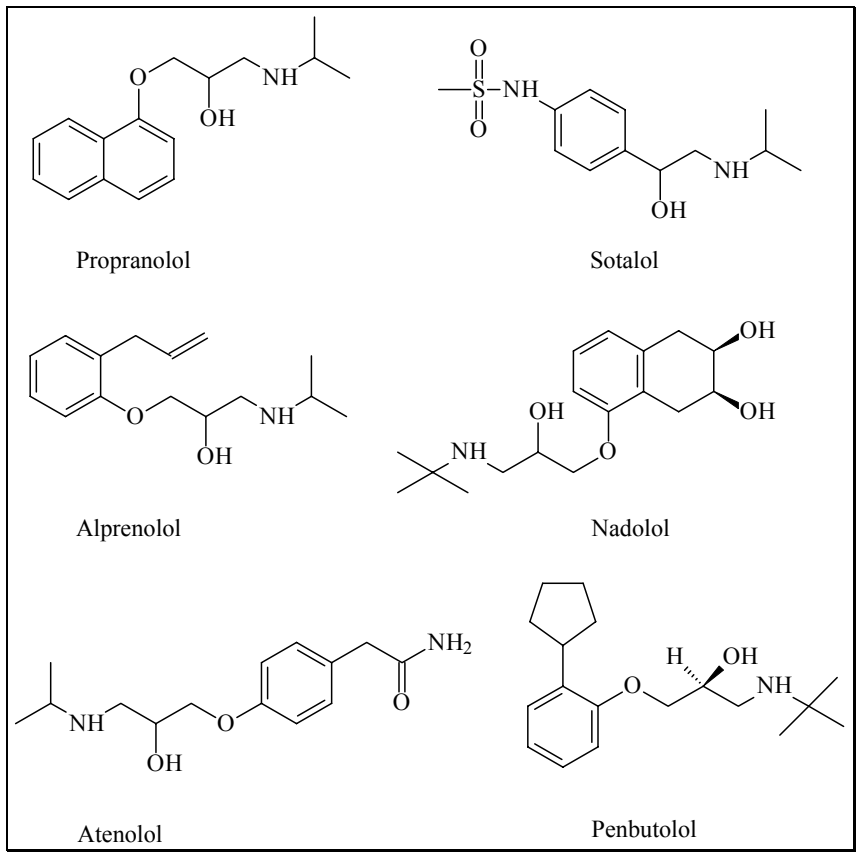

Figure 2: Molecular structures of some selected beta-blockers.

Use as doping agents

Athletes can use diuretics to remove excess water from the body and to lower weight. They are therefore abused in sports with weight categories, such as wrestling, judo and boxing. Diuretics can also be used in an attempt to reduce the concentration of prohibited substances by diluting urine [5] or to counteract the fluid retention as a side effect of excessive use of anabolics [6]. Probenecid is not a diuretic, but a uricosuric agent. In doping analysis probenecid is often included in the screening method for these compounds [7].

Beta-blockers are misused by athletes to reduce anxiety by controlling hand tremor, lowering heart beating rate and blood pressure. They are utilized in sports such as gymnastics, archery and shooting.

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2. Screening for diuretics

Adapted from:

Deventer K, Delbeke FT, Roels K, Van Eenoo P
Screening for 18 diuretics and probenecid in doping analysis by liquid chromatography-tandem mass spectrometry
Biomed. Chromatogr. 2002; **16**: 529.

Abstract

A fast and selective LC-MS$^{(2)}$ method for the screening of 18 diuretics and probenecid in human urine is presented. Analyses were performed on a LCQ-Deca® instrument equipped with an ESI-interface using scan by scan polarity changing. All diuretics and probenecid were separated in less than 20 minutes after liquid/liquid extraction with ethyl acetate.

The LOD for all substances was 100 ng/ml or better. The method was applied to detect diuretics after the oral administration of several drugs including hydrochlorothiazide, bumetanide, spironolactone, furosemide, amiloride, triamterene, chlortalidone and epithizide.

Introduction

Diuretics are banned in sport by WADA and IOC [1]. Diuretics in bodyfluids are often screened for by HPLC-UV [2,3]. However for identification purposes mass spectral data is required. Capillary GC-MS is the standard method for this purpose. However, due to the polar nature of most diuretics a derivatisation step is needed [4]. LC-MS however allows for the direct separation and identification without a preliminary derivatisation step. Recently several methods have been reported for the identification of diuretics by LC-MS$^{(n)}$ [5,6,7].

The aim of this study was to validate a screening method for 18 diuretics and probenecid in human urine following Eurachem validation guidelines [8]. Moreover scan by scan polarity change was used avoiding separate runs in both positive and negative ionisation mode. In addition the method was applied for the detection of diuretics in urine after administration of several therapeutic agents.

Experimental

Chemicals and reagents

Furosemide and piretanide were obtained from Hoechst (Brussels, Belgium), bendroflumethiazide and bumetanide from Leo Pharmaceutical Products Belgium (Brussels), acetazolamide from Cyanamid Benelux (Brussels), chlortalidone and hydrochlorothiazide from Ciba-Geigy (Groot-Bijgaarden, Belgium), epithizide from SMB Technology (Marche-en-Famenne, Belgium), xipamide from Laboratoire CUSI (Brussels), mefruside from Bayer Belgium (Brussels), amiloride from Merck Sharp & Dohme (Brussels), diclofenamide from Alcon-Couvreur (Puurs, Belgium), trichloromethiazide from Merck (Overijse, Belgium), torasemide from Boehringer Mannheim (Brussels), canrenone from Sintesa (Brussels), indapamide from Servier (Neuilly sur Seine, France), triamterene from Smith-Kline (Genval, Belgium), clopamide from Sandoz (Basle, Switzerland), probenecid from Federa (Brussels), ethacrynic acid from Sigma (St. Louis, MO, USA) and 6β-OH-7α-thiomethyl spironolactone, the human metabolite of spironolactone, was from Schering AG (Berlin, Germany).
Analytical grade sodium acetate, potassium carbonate, methanol, diethylether, formic acid and glacial acetic acid were from Merck (Darmstadt, Germany), HPLC grade acetonitrile was from ACROS (Geel, Belgium) and analytical grade ethyl acetate from Panreac (Barcelona, Spain).

Administration studies

The study was performed with four healthy male volunteers aged 25, 28, 33 and 56 years. The study protocol was reviewed and approved by the ethical committee of the institution (UZGent, Project EC/2005-81/sdp). Each volunteer signed an informed consent.
One tablet of the following commercially available drugs was orally taken by healthy male volunteers: Docspirochlor® containing 25 mg spironolactone and 25 mg hydrochlorothiazide, Docpharma (Heverlee, Belgium); Frusamil® containing 40 mg

furosemide and 5 mg amiloride hydrochloride anhydr., Rhône Poulenc Rorer (Brussels); Burinex® containing 1 mg bumetanide, Leo Pharma (Zaventem, Belgium); Dyta-Urese® containing 4 mg epithizide and 50 mg triamterene, SMB laboratories (Brussels); Hygroton® containing 20 mg chlortalidone, Novartis (Brussels) and Torrem® containing 2.5 mg torasemide, Boehringer Mannheim (Brussels).
A blanc urine sample was collected before intake. Samples were quantitatively collected after 1, 2, 4, 6, 9, 12, 24 and 48 h except for Docspirochlor® and Hygroton®, where samples were collected until 120 h after administration.

Sample preparation

The internal standard solution (50 µl mefruside, 20 µg/ml) was added to 2 ml urine, followed by addition of 1 ml sodium acetate buffer (pH 5.2). Liquid-liquid extraction was performed by rolling for 20 min with 4 ml ethyl acetate. After centrifugation the organic layer was transferred into a new tube. To the remaining urine 250 mg of potassium carbonate was added and a second liquid-liquid extraction was performed with 4 ml ethyl acetate. After centrifugation (1200*g*), both organic layers were combined and evaporated until dry under OFN at 40 ºC. The residue was dissolved in 200 µl mobile phase.

Validation

Ten urines were spiked at 8 different levels with 18 diuretics and probenecid. Final concentrations were 200, 100, 50, 20, 10, 5, 2 and 1 ng/ml. The samples were extracted as described above.
The detection limit was defined as the lowest level at which a compound could be identified in all 10 urines with a S/N >3 and a retention time that differs no more than 0,3 min from the retention time in the reference mixture.
Selectivity was tested by analysing several doping agents including beta blockers, narcotics, corticosteroids and anabolic steroids. Several NSAIDs, which are forbidden in equine sports, were tested as well.
Specificity was tested during the validation procedure by analysing ten blank urines.

Apparatus

A Thermo Separation Products (TSP) Model P4000 quaternary pump equipped with a TSP Model AS 3000 autosampler with a 100 µl sample loop and connected to a Finnigan MAT LCQ Deca® mass spectrometer was used.

LC-parameters

A Nucleosil column 3 mm x 100 mm, 5 µm C_{18} protected with a guard column (both from Varian, Sint-Katelijne-Waver, Belgium) were used for chromatographic separation.
The mobile phase consisted of 1% acetic acid (solution A) and acetonitrile (solution B). Gradient elution at a flow rate of 0.3 ml/min was as follows: 85% A for 2 min, linear to 45% in 10 min, linear to 35% in 8 min followed by an increase to 85% in 2 min with 8 min equilibration before the next injection. Total run time: 30 min.

MS-parameters

Ionisation of analytes was carried out using ESI. The capillary temperature was maintained at 335 ºC, the ion source voltage was set at 5000 V and the nebulizer gas at 80 units. The capillary voltage was 44 V in positive mode and -8 V in negative ionisation mode.

Results and discussion

Extraction procedure

Different methods have been published for the detection of diuretics in urine using solid phase [9,10,11] and liquid-liquid extraction [12,13]. Until now analysis with HPLC-UV required an extra clean up step with lead acetate after acidic extraction [14,15]. Using the LC-MS method described here, the clean up step could be omitted.

The choice for mefruside as internal standard was supported by the fact that after administration of mefruside to humans less than 1 % of the dose was found as unchanged drug in urine [8].

MS tuning

Direct infusion was used for optimization of the detection parameters using APCI and ESI. Highest sensitivity was observed with ESI.

As diuretics cover a wide range of substances the optimal ionisation mode however can differ. While basic compounds (e.g. amiloride and triamterene) preferably form $[M+H]^+$ cations, ionisation of acidic compounds (e.g. furosemide, thiazides) results in $[M-H]^-$ anions.

Infusion was used to check for the presence of $[M+H]^+$ or $[M-H]^-$ and product ions. All compounds were tested in positive and negative mode. Therefore a solution of 5 µg/ml, producing a fairly distinguished protonated or deprotonated ion for each compound, was directly infused into the mass spectrometer and the different MS parameters were optimized and saved in a tune file which was used in the screening method.

To test the influence of solvent mixtures on the sensitivity, several combinations were evaluated. No significant difference in signal to noise ratio was found between CH_3OH/CH_3COOH 1%, CH_3OH/HCOOH 1% and CH_3CN/HCOOH 1%.

The decision whether a compound would be analysed in full scan MS or in MS^2 depended on the signal to noise ratio obtained after analysis of extracts from urine spiked at 100 ng/ml. For substances with low signal to noise ratios at this concentration (acetazolamide, clopamide, chlorthalidone, spironolactone metabolite and indapamide) MS^2 resulted in better sensitivity.

The relative retention times and the ions monitored in the screening procedure are presented in Table 1. The collision energy is indicated when appropriate.

For compounds determined in MS/MS, the isolation width was set at 3.0 and an activation Q of 0.250 was applied.

Table 1: RT, screening mode and diagnostic ions in ESI.

Substance	RT (min)	MW	IM	$MS^{(n)}$ (CE) (PI)	Diagnostic ions
acetazolamide	3.08	222	+	MS^2 (27) (223)	181
amiloride	4.21	229	+	MS	230
bendroflumethiazide	14.64	421	-	MS	420
bumetanide	15.37	364	-	MS	363
canrenone	18.07	340	+	MS	341
chlorthalidone	9.26	321	+	MS^2 (32) (321)	240, 304
clopamide	102	345	+	MS^2 (31) (346)	250
diclofenamide	8.57	305	-	MS	303, 305
ethacrynic acid	16.27	303	-	MS	301
furosemide	12.36	329	-	MS	329
hydrochlorothiazide	3.48	297	-	MS	296
indapamide	12.92	365	+	MS^2 (33) (366)	348, 285
mefruside (IS)	13.45	382	-	MS	381
piretanide	14.48	362	-	MS	361
probenecid	16.09	285	-	MS	284
spironolactone metabolite	14.76	386	+	MS^2 (33) (405)	339, 357
torasemide	12.63	348	-	MS	347
triamterene	9.15	253	+	MS	254
trichloromethiazide	10.79	380	-	MS	380
xipamide	14.95	354	-	MS	353

RT: retention time, MW: molecular weight, IM: ionisation mode, CE: collision energy, PI: precursor ion

Validation

All diuretics could be detected at a level of 100 ng/ml. The detection limits are given in Table 2. Ion chromatograms of some selected diuretics obtained after analysis of a control urine spiked at 100 ng/ml are given in Figure 1.

Table 2: Urinary detection limits for diuretics and probenecid

Substance	(ng/ml)	Substance	(ng/ml)
torasemide	50	acetazolamide	50
indapamide	50	hydrochlorthiazide	100
bendroflumethiazide	50	amiloride	100
piretanide	100	triamterene	20
spironolactone metabolite	100	diclofenamide	100
xipamide	20	chlortalidone	10
bumetanide	100	clopamide	2
probenecid	20	trichloromethiazide	50
etachrynic acid	50	furosemide	50
canrenone	50	mefruside*	-

*internal standard

The described method seems to be very selective as no interferences were detected when other doping products including beta-blockers, narcotics, corticosteroids and anabolic steroids were analysed. In addition, NSAIDs did not interfere with the analysis.

Specificity was satisfactory as no interfering substances at the appropriate retention times were found when 10 blank urines were analysed.

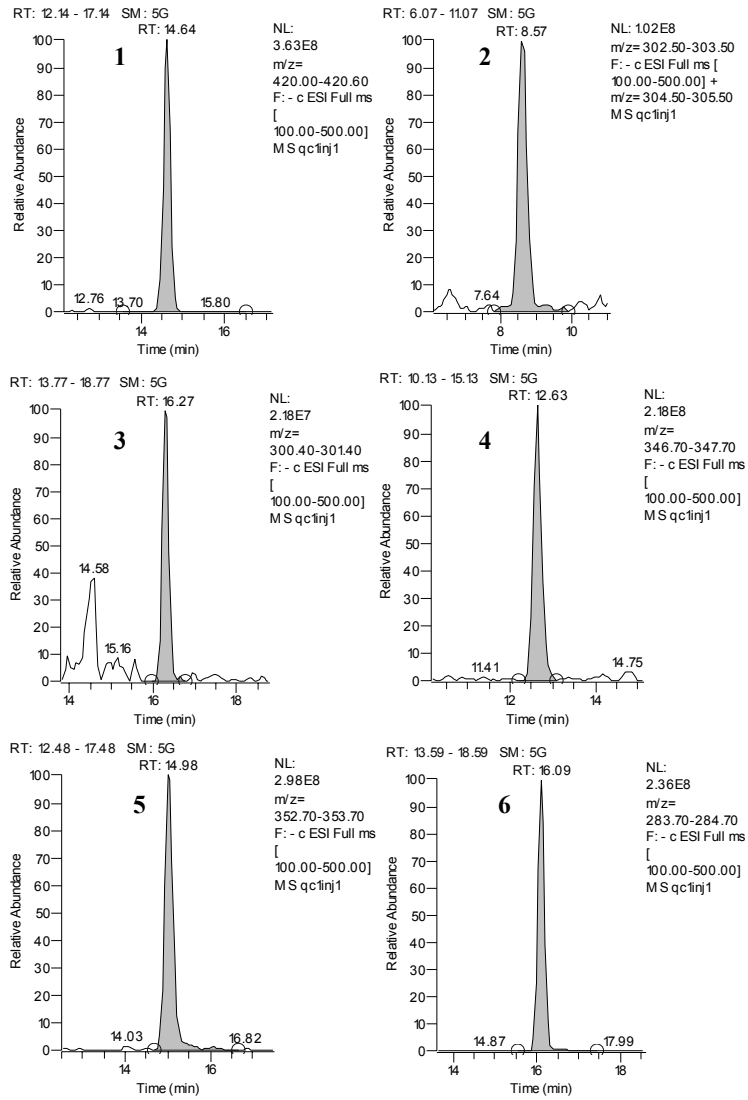

Figure 1: Quality control urine spiked at 100 ng/ml with bendroflumethiaizde (1), diclofenamide (2), etachrynic acid (3), torasemide (4), xipamide (5) and probenecid (6).

Administration studies

Most diuretics are excreted unchanged in urine [4]. Hence, screening for diuretics in human urine is mainly focused on the detection of the parent compound. All compounds, except spironolactone, could be detected in the excretion urines by the presence of the administered drug. Examples of ion chromatograms are presented in Figure 2.

Spironolactone is extensively and fastly metabolised to different compounds [16]. Beta-OH-7-α- thiomethyl spironolactone and canrenone are the most important metabolites. Hence both compounds were used to detect the use of spironolactone. Both metabolites could be detected in the urine sample 4 h after administration (Figure 2).

Seven out of 10 diuretics could already be detected from one hour our onwards after intake (Table 3). Hydrochlorothiazide can be detected for more than 48 h and chlortalidone until 96 h after administration of 50 mg. For compounds administered in low doses (bumetanide and torasemide) detection times were sufficiently long to assess doping abuse.

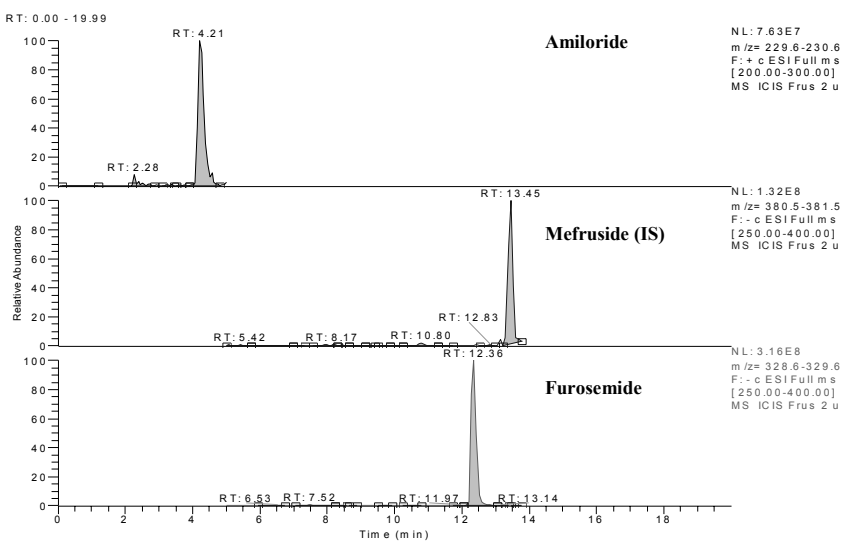

Figure 2(a)

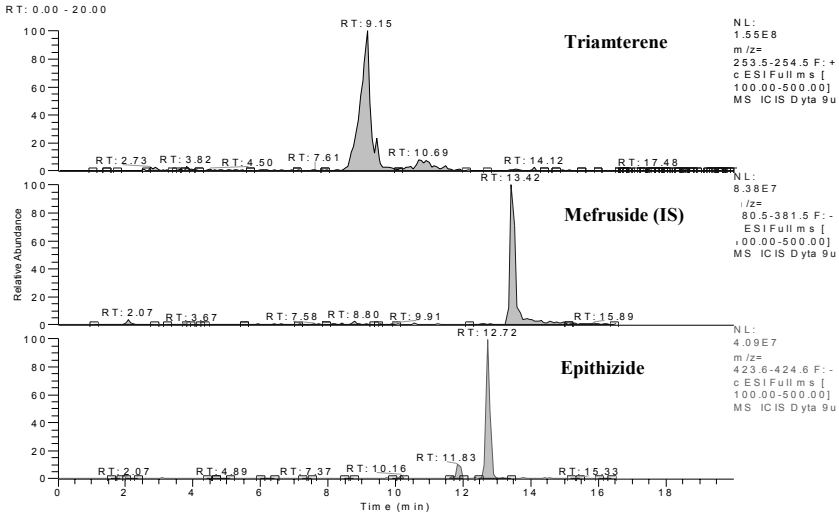

Figure 2(b)

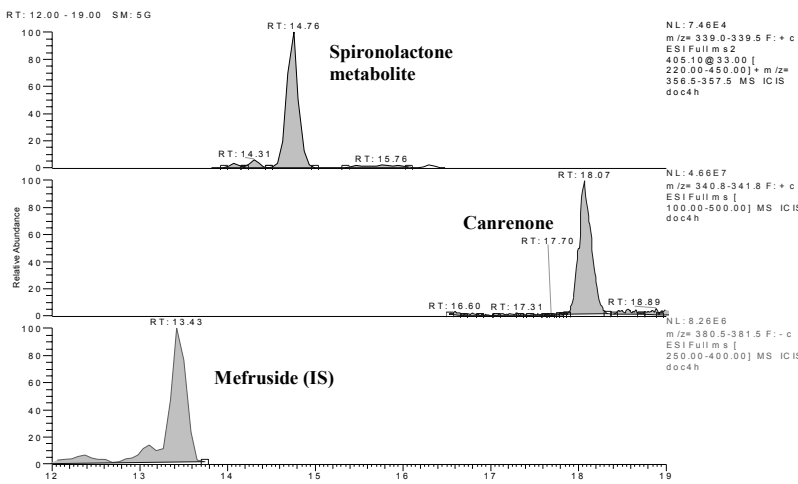

Figure 2(c)

Figure 2: Ion chromatograms obtained after the intake of (a) Frusamil®, 2h sample; (b) Dyta-Urese®, 9h sample; (c) Docspirochlor®, 4h sample.

Table 3: Detection times for several diuretics after administration of a single dose.

Substance	Dose	Traceability
spironolactone	25 mg	2-9 h* / 2-30 h**
hydrochlorothiazide	25 mg	1-60 h
furosemide	40 mg	1-48 h
amiloride	5 mg	2-48 h
bumetanide	1 mg	1-9 h
torasemide	2.5 mg	1-9 h
epithizide	4 mg	2-30 h
triamterene	50 mg	2-36 h
chlortalidone	50 mg	1-96 h

* metabolite β-OH-7-α- thiomethyl spironolactone

** metabolite canrenone

Conclusion

The present work showed that 18 diuretics and probenecid in urine samples can be analysed in a single HPLC run, based on LC-ESI-$MS^{(2)}$ with scan to scan polarity change.

Detection limits were at least 100 ng/ml.

Administration studies indicated that the described method is sufficiently sensitive to detect diuretics in real samples. In addition, typical isotope clusters due to the presence of chlorine in some diuretics, can be helpful for identification purposes.

Although full scan MS-spectra are useful for the detection of the analytes based on their molecular ions, they do not provide sufficient structural information for unambiguous identification. Hence, tandem mass spectrometry is needed to obtain additional structural information for confirmation of suspected samples in doping analysis.

Acknowledgements

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Grants by the Flemish Ministry of Health (KD and PVE) are greatly acknowledged.

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3. Screening for beta-blockers

Adapted from:

Deventer K, Van Eenoo P, Delbeke FT
Simultaneous determination of beta-blocking agents and diuretics in doping analysis by liquid chromatography mass spectrometry with scan-to-scan polarity switching *Rapid Commun. Mass Spectrom.* 2005; **19**: 90.

Abstract

The doping list of prohibited compounds has changed since WADA took over the fight against doping in 2004 from the IOC and was extended with new compounds. Hence the existing screening methods needed to be evaluated.
A previously described method for the screening of 18 diuretics and probenecid was substantially extended with 21 beta-blockers and 8 other diuretics allowing simultaneous determination of diuretics and beta-adrenergic blocking agents in human urine. Analysis was performed using an ion trap instrument equipped with an electrospray ionisation (ESI) interface after liquid/liquid extraction with ethyl acetate. Both full scan MS and full scan MS^2 were applied in combination with scan to scan polarity changing.
The analytical run time was less than 30 minutes and all compounds were well resolved. The detection limits for the diuretics were between 5 and 100 ng/ml and between 5 and 500 ng/ml for the beta-adrenergic blocking agents.

Introduction

Beta-blockers are on the list of prohibited substances published by WADA [1]. Despite the recent publication of sensitive screening methods for the detection of beta-blockers with LC-MS [2,3], GC-MS is still a preferred method for their detection in doping analysis [4-8]. For beta-blockers a derivatisation step is required when GC-MS is used. Several derivatisation agents have been proposed: MSTFA, MBTFA and MBHFB [9,10]. Unfortunately, problems with reproducible derivatisation of beta-blockers have been reported [2]. When WADA took over the fight against doping from the IOC in 2004, the list of beta-blockers was substantially extended. Including these new beta-blockers in our existing GC-MS screening method was not very successful and a new method covering all beta-blockers was therefore urgently needed. Hence it was decided to include the full list of beta-blockers published by WADA in our routine LC-MS screening method for the diuretics [11]. The transfer from GC-MS to LC-MS will omit the development of an extra screening method as well as a time consuming derivatisation step. In addition eight new diuretics were added to the existing method.

Two other compounds, strychnine (a stimulant) and mesocarb hydroxysulphate (the major metabolite of mesocarb) were also included since these compounds show good sensitivity when analysed by LC-MS [12-14].

Experimental

Chemicals and reagents

Beta-blockers obtained as reference substances were: acebutolol from Rhone-Poulenc (Brussels, Belgium), alprenolol from Astra Chemicals (Holstein, Germany), atenolol and propranolol from ICI (Kortenberg, Belgium), betaxolol from Synthelabo (Brussels), labetolol from Glaxo (Brussels), metoprolol from Ciba-Geigy (Groot-Bijgaarden, Belgium), nadolol from Squibb (Braine l'Alleud, Belgium), oxprenolol from CIBA (Dilbeek, Belgium), pindolol from Sandoz (Vilvoorde, Belgium), sotalol from Pfizer (Brussels), timolol from MSD (Brussels), penbutolol from Thomson (London, United Kingdom), bisoprolol from Merck (Darmstadt, Germany), mepindolol from Schering (Machelen, Belgium) and carvedilol from Roche (Mannheim, Germany).
levobunolol (*l*-bunolol) and esmolol were a kind gift from the South African doping control laboratory. Carteolol was a gift from the Portugese doping control laboratory. The following substances were extracted from therapeutical preparations: celiprolol (Selectol®, Pharmacia, Brussels) and metipranolol (Beta-Ophtiole®, Tramedic, Sint-Niklaas, Belgium).
Hydroflumethiazide was obtained from Leo Pharmaceutical Products Belgium (Brussels), cyclopenthiazide from Ciba-Geigy, epitizide from SMB Technology (Marche-en-Famenne, Belgium), mebutizide from Sintesa (Brussels), spironolactone from Schering AG (Berlin, Germany), bemitizide from BYK Belga (Machelen, Belgium), polythiazide from Pfizer (Brussels) and althiazide from Continental Pharma (Brussels).
Strychnine was obtainend from Sigma (Bornem, Belgium) and mesocarb was a gift from the Moscow doping control laboratory.
Analytical grade sodium acetate, potassium carbonate, methanol, diethylether, glacial acetic acid were from Merck (Darmstadt, Germany), HPLC grade acetonitrile was from

ACROS (Geel, Belgium) and analytical grade ethyl acetate was from Panreac (Barcelona, Spain).

Sample treatment

An internal standard solution (50 µl mefruside, 20 µg/ml) was added to 2 ml of urine, followed by addition of 1 ml sodium acetate buffer (pH 5.2). Liquid-liquid extraction was performed by rolling for 20 min with 4 ml ethyl acetate. After centrifugation (1200g) the organic layer was transferred into a new tube. To the remaining urine 250 mg of potassium carbonate was added and a second liquid-liquid extraction was performed with 4 ml ethyl acetate. After centrifugation, both organic layers were combined and evaporated until dry under OFN at 40 ºC. The remaining residue was dissolved in 200 µl of the initial mobile phase.
Extraction recovery of the beta-blockers was tested as well. For this purpose negative urine samples (n= 6) were spiked at 500 ng/ml and extracted together with non spiked negative urine samples (n=6). The extracts of the non spiked urine samples were then spiked at 500 ng/ml simulating a 100% recovery. Both sets of samples were evaporated and analysed with the described LC-MS method. The obtained peak areas of the two sets of samples were compared.

Validation

The validation was carried out following Eurachem validation guidelines [15].
Ten human urine samples, declared negative after routine doping analysis, were spiked at 9 different levels. Final concentrations were 500, 250, 100, 50, 20, 10, 5, 2 and 1 ng/ml. The samples were extracted as described above.
The detection limit was defined as the lowest level at which a compound could be identified in all 10 urines, with diagnostic ions present with a S/N ratio greater than 3 and a retention time that differs no more than 0.3 min from the compounds in the reference mixture. Selectivity was tested by analysing several doping agents which are routinely screened for including narcotics, corticosteroids, anabolic steroids and

stimulants. NSAIDs, forbidden in equine sports, were tested as well. Concentrations of these substances were 1 µg/ml.
Specificity was tested during the validation procedure. Ten blank urines were extracted and analysed as described above.

Apparatus

A TSP Model P4000 quaternary pump equipped with a TSP Model AS 3000 autosampler with a 100 µl sample-loop and connected to a Thermo Electron LCQ-Deca® mass spectrometer was used.

Chromatography

A Nucleosil C18 column 3 mm x 100 mm, 5 µm and a guard column 10 x 2 mm (both from Varian, Sint-Katelijne-Waver, Belgium) were used for the chromatographic separation.
The mobile phase consisted of 1% acetic acid (solution A) and acetonitrile. Gradient elution at a flow rate of 0.3 ml/min was as follows: 85% A for 2 min, linear to 45% in 10 min, linear to 35% in 8 min followed by an increase to 85% with 10 min equilibration time before the next injection. Total run time: 30 minutes.

MS parameters

Ionisation of analytes was carried out using electrospray ionisation. The capillary temperature was maintained at 300 ºC, the ion source voltage was set at 5000 V and the nebulizer gas (nitrogen) was set at 80 units. The make up gas (nitrogen) was set at a value of 30. The capillary voltage was 10 V in positive mode and -4 V in negative ionisation mode.
When MS^2 was applied the isolation width was set at 3.0, the activation q at 0.250 and the activation time at 30 ms. An exception was made for acetazolamide for which a q value of 0.3 and an activation time of 70 ms was applied.

Results and Discussion

Method development

Beta-blockers show less differences in pKa values then diuretics. Most beta-blockers have a secondary or tertiary amine function and can be extracted at the basic pH used for the extraction of the basic diuretics. Indeed, for most beta-blockers good extraction recoveries were observed (Table 1). Lowest recoveries were observed for mepindolol and sotalol and highest recovery was obtained for oxprenolol.

Table 1: Extraction recoveries for beta-blockers

Substance	Recovery (%)	Substance	Recovery (%)
Acebutolol	92.4 ± 24.2	Mepindolol	42.8 ± 6.7
Alprenolol	93.7 ± 12.7	Metipranolol	90.4 ± 2.0
Atenolol	81.7 ± 4.3	Metoprolol	89.1 ± 5.4
Betaxolol	94.2 ± 16.5	Nadolol	90.6 ± 2.3
Bisoprolol	97.7 ± 5.4	Oxprenolol	98.4 ± 2.5
Carteolol	90.1 ± 8.4	Penbutolol	89.2 ± 8.3
Carvedilol	73.9 ± 4.9	Pindolol	81.1 ± 3.4
Celiprolol	90.4 ± 3.7	Propranolol	97.6 ± 5.7
Esmolol	93.7 ± 2.6	Sotalol	42.9 ± 6.9
Labetolol	95.6 ± 7.9	Timolol	93.4 ± 5.1
Levobunolol	85.1 ± 2.6		

[a] values are presented as mean ± standard deviation (n=6), concentrations 500 ng/ml.

Beta-blockers exhibit great differences in phase I metabolism [16]. Hence the amount of administered drug excreted unchanged in the urine can vary from 76 % for carteolol to less than 1% for carvedilol and propranolol [16]. Despite the poor urinary excretion of some beta-blockers, they are generally screened for by the parent drug [2-5]. Indeed, preliminary LC-MS experiments with excretion urines and samples declared positive by

our GC-MS screening method showed that all beta-blockers could be detected by the parent compound.

Peak tailing in reversed phase HPLC is particularly prevalent when silanol groups interact with basic compounds, particularly amines. Since all beta-blockers contain amine functions broad tailing peaks were observed. However detection of beta-blockers and diuretics in doping analysis is not quantitative and peak shape is of minor importance. Reproducibility in retention times for beta-blockers was also less satisfactory than for the diuretics.

Flow injection analysis was performed in order to determine diagnostic ions for the newly added compounds. For each tested compound a solution of 5 µg/ml was infused at a flow rate of 10 µl/min.

As expected for the acidic diuretics (e.g. hydroflumethiazide) $[M-H]^-$ and $[M+CH_3COO]^-$ ions were observed. For the neutral diuretic spironolactone a positively charged $[M+H]^+$ion was observed.

Beta-blockers contain a basic group which can easily be protonated. Very abundant protonated molecular ions $[M+H]^+$ were observed in ESI. No deprotonated molecular ions were detected in negative ionisation mode.

The amine function in strychnine resulted in an abundant protonated molecular ion $[M+H]^+$.

Diagnostic ions monitored in the screening method are presented in Tables 2 and 3.

The lenses and skimmer voltages were automatically optimised, in positive mode on the protonated molecular ion of carvedilol and in negative mode on the protonated molecular ion of the internal standard mefruside.

Table 2: Retention time, ionisation mode, diagnostic ions and LODs for diuretics, strychnine, probenecid and mesocarb hydroxysulfate.

Substance	RT (min)	MW	IM	MS^2 (CE) (PI)	Diagnostic Ions	LOD (ng/ml)
acetazolamide	3.2	222	-	MS^2 (32) (221)	83	50
althiazide	12.7	383	-	MS	382	50
amiloride	3.5	229	+	MS^2 (31) 230	213	50
bemithiazide	14.5	401	-	MS	400	25
bendroflumethiazide	14.8	421	-	MS	420	10
bumetanide	15.8	364	-	MS	363	25
canrenone	18.6	340	+	MS^2 (28) (341)	187	5
chlorthalidone	9.5	338	-	MS^2 (36) (337)	319	25
clopamide	10.7	345	-	MS^2 (35) (344)	308	10
cyclopenthiazide	14.9	379	-	MS	378	10
diclofenamide	8.9	305	-	MS	303, 305	50
epitizide	13.2	425	-	MS	424	10
ethacrynic acid	16.4	302	-	MS	301	10
furosemide	12.8	330	-	MS	329	50
hydrochlorothiazide	4.4	297	-	MS	296	100
hydroflumethiazide	7.2	331	-	MS	330	50
indapamide	13.4	365	-	MS^2 (33) (364)	216, 233	25
mebutizide	15.4	381	-	MS	380	100
mefruside (ISTD)	14.0	382	-	MS^2(30) (381)	345	-
mesocarb hydroxy sulphate	10.6	418	+	MS	419	-
piretanide	14.9	362	-	MS	361	50

Table 2: continued

Substance	RT (min)	MW	IM	MS^2 (CE) (PI)	Diagnostic Ions	LOD (ng/ml)
polythiazide	14.7	439	-	MS	440	10
probenecid	16.2	285	-	MS	284	25
spironolactone	17.8	415	+	MS^2 (28) (341)	187	10
spironolactone metabolite	15.1	404	+	MS^2 (30) (405)	339, 357	100
strychnine	8.5	334	+	MS	335	25
torasemide	12.7	348	-	MS	347	25
triamterene	7.9	253	+	MS	254	10
trichlormethiazide	10.9	380	-	MS	306	25
xipamide	15.2	354	-	MS	353	25

RT: retention time, MW: molecular weight, IM: ionisation mode, CE: collision energy, PI: precursor ion

Table 3: Retention time, diagnostic ions and LODs for the beta-blockers. All beta-blockers were analysed in full scan MS, positive mode.

Substance	RT	MW	Diagnostic Ion	LOD (ng/ml)
acebutolol	9.2	336	337	25
alprenolol	14.2	249	250	10
atenolol	2.6	266	267	50
betaxolol	14.7	307	308	25
bisoprolol	12.5	325	326	10
carteolol	5.6	292	293	10
carvedilol	19.3	406	407	10
celiprolol	11.3	379	380	5
esmolol	11.0	295	296	5
labetolol	12.2	328	329	10
levobunolol	10.2	291	292	5
mepindolol	8.9	262	263	500
metipranolol	13.5	309	310	25
metoprolol	9.8	267	268	10
nadolol	5.5	309	310	10
oxprenolol	11.8	265	266	10
penbutolol	22.1	291	292	5
pindolol	7.0	248	249	25
propranolol	13.8	259	260	10
sotalol	3.0	272	273	50
timolol	8.9	316	317	25

RT: retention time, MW: molecular weight, IM: ionisation mode

Scan to scan polarity changing

Due to the acidic nature of most diuretics, negative ionisation is generally prefered (Table 2). For the basic diuretics, beta-blockers, strychnine and mesocarb hydroxysulphate however positively charged ions are formed.

Hence, positive and negative scan events are necessary to cover all compounds in the screening method. In addition coelution of positively and negatively charged ions makes scan to scan polarity switching unavoidable. Before the introduction of robust instruments scan to scan polarity switching was technically difficult to perform and two consecutive runs in both ionisation modes were necessary [18]. Soon after the introduction of reliable and fast polarity switching instruments reproducible scan to scan polarity switching was reported [11,12,19].

Although the advantage of simultaneous covering both ionisation modes polarity switching requires a period (approximately 500 ms) in which the instrument is "off" and no scans can be performed. When every compound would be analysed in MS^2 additional scans would be lost because every MS^2 setting is specific for only one substance.

Because polarity switching was a prerequisite of the method to allow for the detection of all compounds, full scan MS mode was chosen rather than full scan MS^2 to keep the number of data points for every compound as high as possible. Nevertheless, when a compound showed insufficient sensitivity in full scan MS, full scan MS^2 was applied to fullfill the WADA MRPL of 250 ng/ml for diuretics and 500 ng/ml for the beta-blockers [20].

Validation

All diuretics could be detected at least at a level of 100 ng/ml. The LODs for the beta-blockers were below 50 ng/ml except for mepindolol (500 ng/ml). The LODs for the diuretics and beta-blockers are presented in Table 2 and 3, respectively.

Ion chromatograms obtained after analysis of a control urine spiked at 100 ng/ml with some selected beta-blockers is presented in Figure 1.

The method seems to be very selective as no interferences were detected when other doping substances including narcotics, corticosteroids, stimulants and anabolic steroids were analysed. In addition, several NSAIDs did not interfere with the analysis.

Specificity was satisfactory as no interfering substances at the appropriate retention times were found when 10 blank urine samples were analysed.

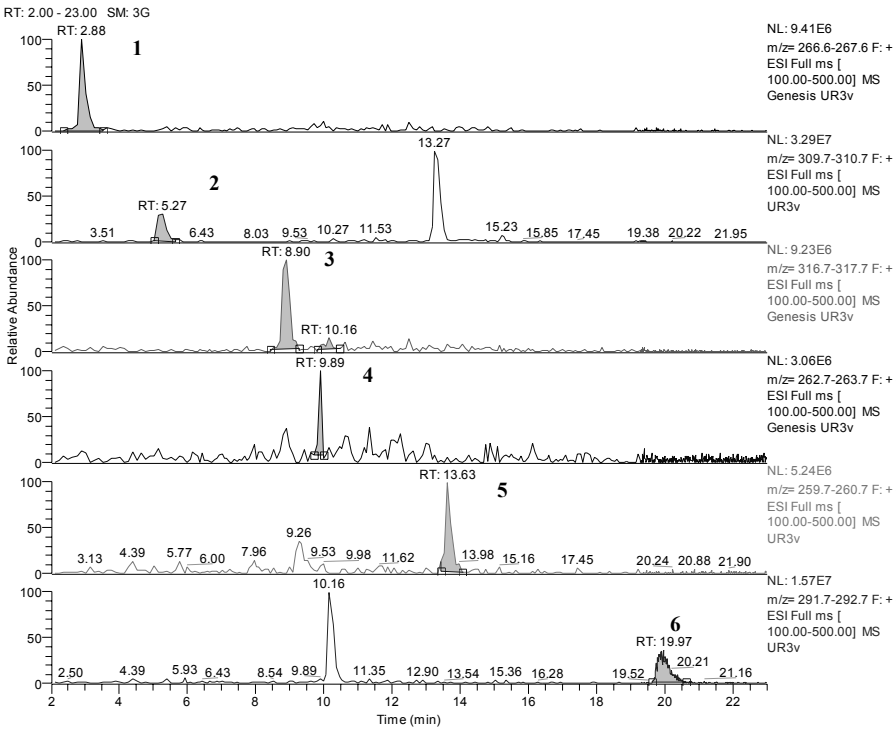

Figure 1: Selected ion chromatograms of a quality control urine spiked at 100 ng/ml (mepindolol at 500 ng/ml) with atenolol (1), nadolol (2), timolol (3), mepindolol (4), propranolol (5), penbutolol (6).

Conclusion

The existing screening method for diuretics in our laboratory was successfully extended and validated with 8 other diuretics and 21 beta-blocking agents. Moreover strychnine and mesocarb hydroxysulphate were included and validated as well. Detection limits were at least 100 ng/ml for diuretics and 500 ng/ml for beta-blockers.

Although full scan LC-MS is a useful tool in the screening of urine samples, this technique often do not provide for the desired mass spectrometric information to declare a sample positive. For confirmation purposes, tandem mass spectrometry will be needed.

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Chapter III: Corticosteroids

1. Introduction

Therapeutic use

Corticosteroids can be divided in two groups: the glucocorticosteroids and the mineralocorticosteroids.
Mineralocorticosteroids affect water and electrolyte balance, i.e. potassium is lost and sodium and water are retained in response to aldosterone, the main endogenous mineralcorticosteroid. The term “glucocorticosteroid” is derived from their action to stimulate the increase of glucose levels for energy [1]. More important is their anti-inflammatory potency. Therefore glucocorticosteroids are frequently used in the short-term treatment of many inflammatory disorders including infections, allergies, skin problems, asthma or arthritis.
The use of large doses can cause an immediate psychoendocrine effects in overcoming tiredness. Prolonged use can result in immunosupression and water retention.
In children, the use of corticosteroids can result in growth impairment [2]. Some therapeutical important corticosteroids are presented in Figure 1.

Use as doping agents

Corticosteroids affect the nervous system, cause euphoria and improve the athlete's ability to concentrate in performance of endurance and power events [3,4]. Moreover, corticosteroids can alleviate pain. As a result of high physical efforts, pain and injuries are related to sport. Corticosteroids are therefore widely used as pain and inflammatory relieving agents.
Because of their widespread use, corticosteroids are subjected to complicated doping regulations. Systemic use (intravenous or intramuscular injections, oral and rectal administration) is forbidden.
Non systemic use including topical application (buccal, nasal, creams and eyedrops) is allowed while other non systemic applications (intra-articular, inhalations) require an abbreviated TUE [5].

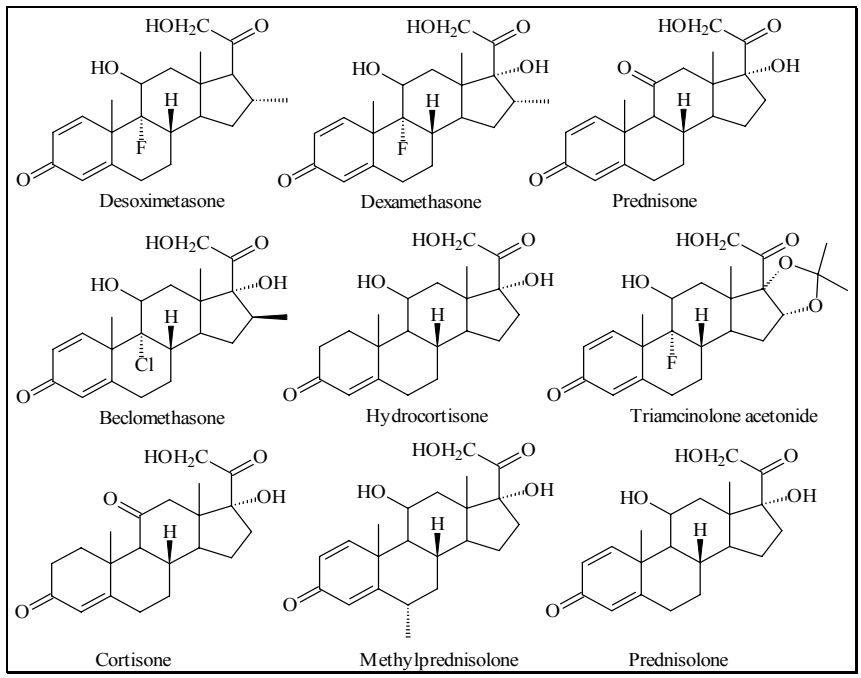

Figure 1: Molecular structure of selected corticosteroids

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2. Screening for corticosteroids

Adapted from:

Deventer K, Delbeke FT.
Validation of a screening method for corticosteroids in doping analysis by liquid chromatography-tandem mass spectrometry.
Rapid Commun. Mass Spectrom. 2003; **17**: 2107.

Abstract

A selective and sensitive method for the screening of 9 corticosteroids in human urine has been validated. Analysis was performed using an ion trap instrument equipped with an ESI interface. All corticosteroids were separated in less than 20 min after liquid/liquid extraction with diethylether. The limit of detection for all substances was 4 ng/ml or lower.
Validation of the chromatographic separation and mass spectrometric identification of mixtures of betamethasone and dexamethasone are also presented.

Introduction

Before the introduction of liquid chromatography - tandem mass spectrometry, detection of corticosteroids was done by GC-MS [1,2]. Selectivity was sufficient, but a time-consuming derivatisation step was necessary. HPLC-UV was not an alternative due to its low sensitivity and specificity. In the past, ELISA was used for the screening of corticosteroids in horse urine in our laboratory, ELISA-positive samples were confirmed by GC-MS. Different ELISA kits were necessary to cover the wide range of corticosteroids resulting in time-consuming and expensive analyses. Therefore a comprehensive method covering a wide range of corticosteroids was needed. LC-MS seemed to be the method of choice for the detection of corticosteroids as it combines selectivity and sensitivity without the need for derivatization.
Recently, several methods have been reported for the identification of corticosteroids in human urine by LC-$MS^{(n)}$ using solid phase extraction [3-5] or filtration [6] as clean-up steps. The aim of the present study was to validate a comprehensive LC-MS screening method using a single liquid-liquid extraction following Eurachem validation guidelines [7]. A time-consuming hydrolysis step [5,6] was omitted. In addition 16α–hydroxyprednisolone, the major metabolite of budesonide [8], was also included in the screening method.
As concurrent use of betamethasone and dexamethasone can lead to the detection of both corticosteroids in the same urine sample, a method for the separation and identification of both isomers was also validated [9,10].

Experimental

Chemicals

Beclomethasone and desoximetasone were bought from Sigma-Aldrich (Bornem, Belgium). Betamethasone and budesonide were a gift from Glaxo-Wellcome (Greenford, Great Britain). Cortisone was purchased from Akzo (Brussels, Belgium) and dexamethasone from Organon (Brussels). Hydrocortisone was obtained from ERFA (Brussels) and methylprednisolone from Pharmacia (Diegem, Belgium). Triamcinolone acetonide was a gift from Labaz (Brussels) and the metabolite of budesonide, 16α–hydroxyprednisolone, was obtained from Astra-Zeneca (Lund, Sweden).
Potassium carbonate p.a., methanol p.a., diethylether p.a. and acetic acid p.a. were obtained from Merck (Darmstadt, Germany). HPLC grade acetonitrile was purchased from ACROS (Geel, Belgium).

Sample preparation

The internal standard solution (50 µl desoximetasone, 1 µg/ml) was added to 5 ml urine, followed by the addition of 1 g K_2CO_3. Liquid-liquid extraction was performed by rolling for 20 min with 5 ml diethylether [9-12]. After centrifugation (1200g) the organic layer was separated and evaporated until dry under OFN at 40 ºC. The remaining residue was dissolved in 200 µl mobile phase
(70/30,1% acetic acid /acetonitrile).

Validation

Screening method
Ten human urine samples declared negative after routine doping analysis were spiked with 7 corticosteroids at 6 different levels. Final concentrations were 8, 4, 2, 1, 0.5, 0.25 and 0.1 ng/ml. The samples were extracted as described above. The LOD was defined as the lowest level at which a compound could be identified in all 10 urines. Two diagnostic ions should be observed with a signal to noise (S/N) ratio greater than 3 and a

retention time that differs by not more then 0.2 min from the RT in the reference mixture. Selectivity was tested by analysing reference mixtures consisting of several doping agents routinely screened for in our laboratory including 17 beta blockers, 25 narcotics, 19 diuretics and 22 anabolic steroids. Eighteen NSAIDs, forbidden in equine sports, were tested as well. Individual concentrations in these reference mixtures were 1 µg/ml. Specificity was tested by analysing 10 blank urines.

Differentiation between betamethasone and dexamethasone

Two sets of 5 urine samples, declared negative after routine doping analysis, were spiked with a mixture of betamethasone and dexamethasone at the LOD. The two sets were extracted and analysed on different days. Chromatographic and mass spectrometric criteria specified by WADA were applied for the identification [13].

Apparatus

A TSP Model P4000 quaternary pump equiped with a TSP Model AS 3000 autosampler with a 100 µl sample-loop and connected to a Finnigan MAT LCQ-Deca® (Thermo Finnigan, San Jose, USA) ion trap mass spectrometer was used.

A Nucleosil C18 column 100 x 3 mm, 5 µm , protected with a guard column 10 x 2 mm (both from Varian, Sint-Katelijne-Waver, Belgium), was used for chromatographic separations. Elution solvents were 1% acetic acid (v/v) in water (Solution A) and acetonitrile (Solution B). Gradient elution at a flow rate of 0.3 ml/min was as follows: 70% A for 5 min, linear gradient to 35% A in 2 min, isocratic for 5 min, followed by 70% A with 8 min equilibration before the next injection. The total run time was 20 min.

Separation of betamethasone and dexamethasone was done as follows: isocratic 25 % B for 22 min followed by a linear gradient to 65 % B in 0.5 min, isocratic for 7.5 min and an equilibration step of 10 min with 25% B. The flow rate was 0.3 ml/min. The total run time was 40 min.

Mass spectral data for the different compounds was obtained by direct infusion of a solution of 5 µg/ml. As corticosteroids show great similarity in structure and chemical

properties a unique tunefile was used for each substance. Ionisation of analytes was achieved using ESI. The capillary temperature was maintained at 300 ºC, the ion source voltage was set at 5000 V and the nebulizer gas (nitrogen) was 80 (arbitrary) units. The auxiliary gas flow was set at 10 units. The capillary voltage was -18 V in the negative ionisation mode and 4 V in the positive ionisation mode. In both MS^2 and MS^3 modes the isolation width was set at 3.0 and an activation q of 0.250 was applied.

Results and Discussion

Sample preparation

Conjugation of synthetic corticosteroids is hampered by the presence of a double bond between the carbons at C1 and C2 and/or a fluorine at C9 [14] as a result corticosteroids can be detected in human urine without hydrolysis [3,4], omiting a time consuming hydrolysis step. Alkaline extraction with diethylether is routinely used in our laboratory for the extraction of anabolic agents. As corticosteroids are chemically related to anabolic agents an extraction method using potassium carbonate was applied.

MS tuning

Different LC-MS methods have been reported for the detection of corticosteroids using APCI and ESI in both positive and negative modes [10,12,15]. A choice had to be made between APCI and ESI. The highest sensitivity was observed in ESI negative ionisation mode with base peaks corresponding to $[M + CH_3COO]^-$ adduct ions. $[M-H]^-$ ion were not observed. Because full scan LC-MS spectra contain few diagnostic ions, tandem mass spectrometry was applied. Most compounds exhibited fragment ions $[M-H]^-$ and $[M-H-CH_2O]^-$ in the MS^2-spectra of the acetate adduct ions [16]. For triamcinolone acetonide loss of the CH_2O moiety was not observed, but loss of the ketal moiety on the C16 and C17 position resulted in an abundant ion at m/z 375 $[M-H-(CH_3)_2CO]^-$. Additional loss of hydrofluoric acid and water was also noticed (Figure 1).

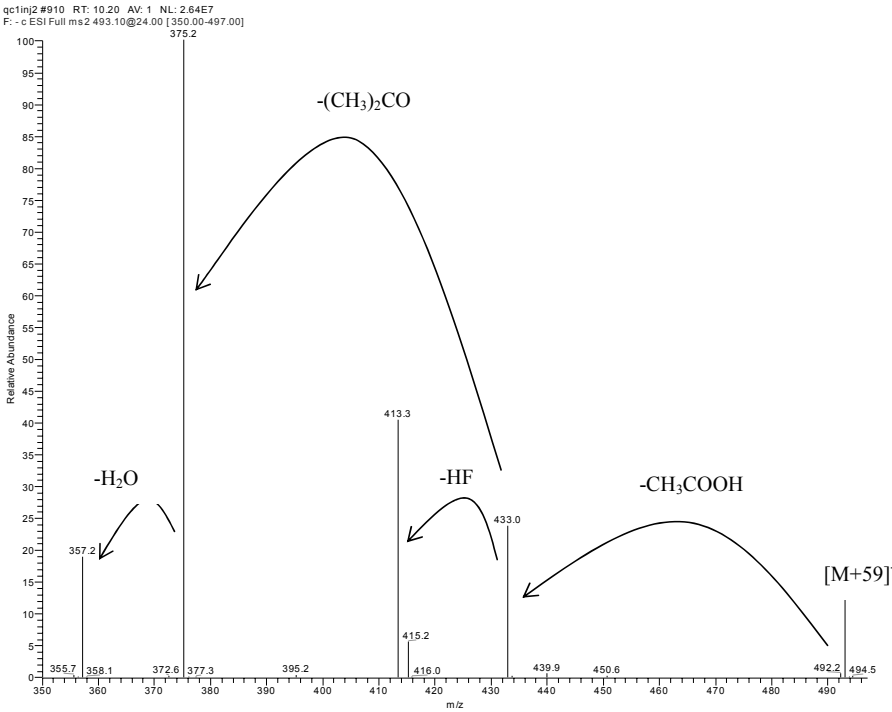


Figure 1: MS^2 spectrum of triamcinolone acetonide (precursor ion m/z 493).

In the MS^2 experiments two diagnostic ions were selected (Table 1). Negative ionisation tandem mass spectrometry for 16α–hydroxyprednisolone only resulted in the product ion $[M-H]^-$, consequently positive ionisation tandem mass spectrometry was used for this compound. The monitored ions are presented in Table 1.

Table 1: Ionisation mode and diagnostic ions

Substance	M_w	Ionisation mode	CE	Precursor ion	Diagnostic ions
16α–hydroxy-prednisolone	376	MS^2 (+) ESI	20.0	377	341, 323
Hydrocortisone	362	MS^2 (-) ESI	22.5	421	361, 331
Cortisone	360	MS^2 (-) ESI	21.0	419	359, 329
Methylprednisolone	374	MS^2 (-) ESI	22.5	433	343, 373
Betamethasone	392	MS^2 (-) ESI	21.5	451	361, 391
Dexamethasone	392	MS^2 (-) ESI	21.5	451	361, 391
Beclomethasone	408	MS^2 (-) ESI	21.5	467	377, 407
Triamcinolone acetonide	434	MS^2 (-) ESI	24.0	493	375, 413
Desoximetasone (IS)	376	MS^2 (-) ESI	24.0	435	375, 355
Budesonide	430	MS^2 (-) ESI	22.0	489	357, 339

M_W: molecular weight (Da), CE: collision energy (% of maximum)

Validation of the screening method

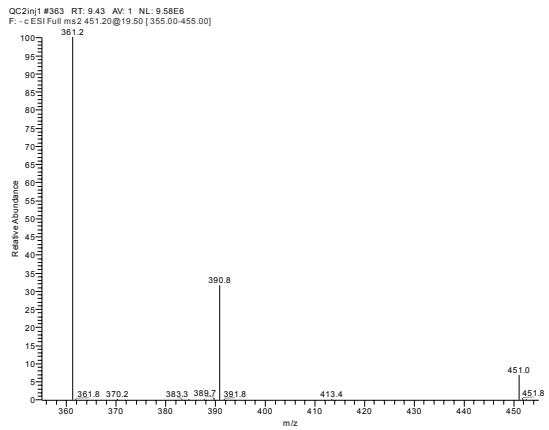
The method seemed to be very sensitive as all corticosteroids could be detected at 4 ng/ml or lower (Table 2). The method is also selective as no interferences were found when other doping agents, including beta-blockers, narcotics, diuretics and anabolic steroids were analysed. In addition, several NSAIDs did not interfere. The specificity was satisfactory as no interfering substances at the appropriate retention times were found when 10 blank urines were analysed.

Table 2: LODs for the corticosteroids

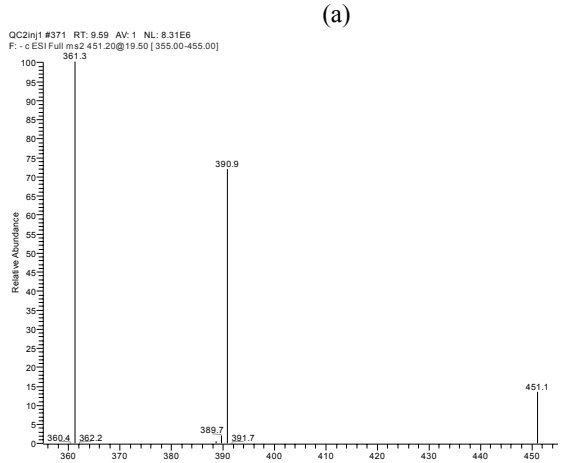
Substance	LOD (ng/ml)
Desoximetasone (IS)	-
Triamcinolone acetonide	0.5
Dexamethasone	1
Betamethasone	2
Budesonide	4
16 α-Hydroxyprednisolone	4
Methylprednisolone	2
Beclomethasone	0.5
Hydrocortisone	endogeneous
Cortisone	endogeneous

Differentiation between betamethasone and dexamethasone

Although LC-MS is the method of choice for the detection of corticosteroids, optimalisation of the chromatographic parameters requires compromises between analysis time and resolution. Although chromatography can be not sufficient to separate all compounds in a short run, mass spectrometric detection can provide for the desired selectivity. However, this was not the case for the partially coeluting corticosteroids dexamethasone and betamethasone [3,5,11]. Betamethasone and dexamethasone are isomers differing only in the orientation of the methyl group at C16. As expected the MS^2 spectra obtained using infusion contained the same fragment ions, $[M-H]^-$ at m/z 391 and $[M-H -CH_2O]^-$ at m/z 361 (Figure 2). However, when MS^3 with m/z 361 as precursor ion is applied, the difference between betamethasone and dexamethasone is more pronounced (Figure 3). For betamethasone the ion m/z 307 is the most abundant while for dexamethasone the ion m/z 345 is the most intense.



(a)



(b)

Figure 2: MS^2 spectra of the $[M+CH_3COO]^-$ adduct ions of (a) betamethasone and (b) dexamethasone

In addition, better chromatographic separation was achieved by applying alternative (isocratic) conditions, resulting in a difference in retention time of approximately 1 min (Figure 3). The results for the two sets of 5 urines spiked with a mixture of betamethasone and dexamethasone at their respective LODs are presented in Table 3. Differences in RRT were significant (Student t-test) while the intensities of the diagnostic ions differ by not more than 10 % absolutely or 25 % relatively, whichever is

the greater [13], from the respective intensities in the reference mixture. TICs and mass spectra of a reference mixture and a spiked urine sample are presented in Figure 3.

Table 3: RRT and relative intensities of the diagnostic ions for betamethasone and dexamethasone

		reference	spike1	spike2	spike3	spike4	spike5	Mean	SD
Set 1				Betamethasone					
RRT		0.733	0.733	0.736	0.735	0.734	0.734	0.734	0.0011
Intensities	m/z 307	100	100	100	100	100	100	100	0
	m/z 325	34	35	34	33	32	35	33.8	1.1
	m/z 345	89	92	84	84	82	88	86	4
				Dexamethasone					
RRT		0.769	0.771	0.776	0.768	0.767	0.773	0.771	0.0037
Intensities	m/z 307	62	61	69	60	75	67	66.4	6.1
	m/z 325	23	19	28	20	22	28	23.4	4.3
	m/z 345	100	100	100	100	100	100	100	0
Set 2				Betamethasone					
RRT		0.715	0.721	0.721	0.718	0.715	0.717	0.718	0.0026
Intensities	m/z 307	100	100	100	100	100	100	100	0
	m/z 325	31	28	29	28	28	29	28.4	0.5
	m/z 345	61	59	59	63	60	58	59.8	1.9
				Dexamethasone					
RRT		0.753	0.758	0.755	0.759	0.755	0.752	0.756	0.0028
Intensities	m/z 307	93	93	96	96	93	87	93	3.7
	m/z 325	32	27	28	29	29	37	30	4
	m/z 345	100	100	100	100	100	100	100	0

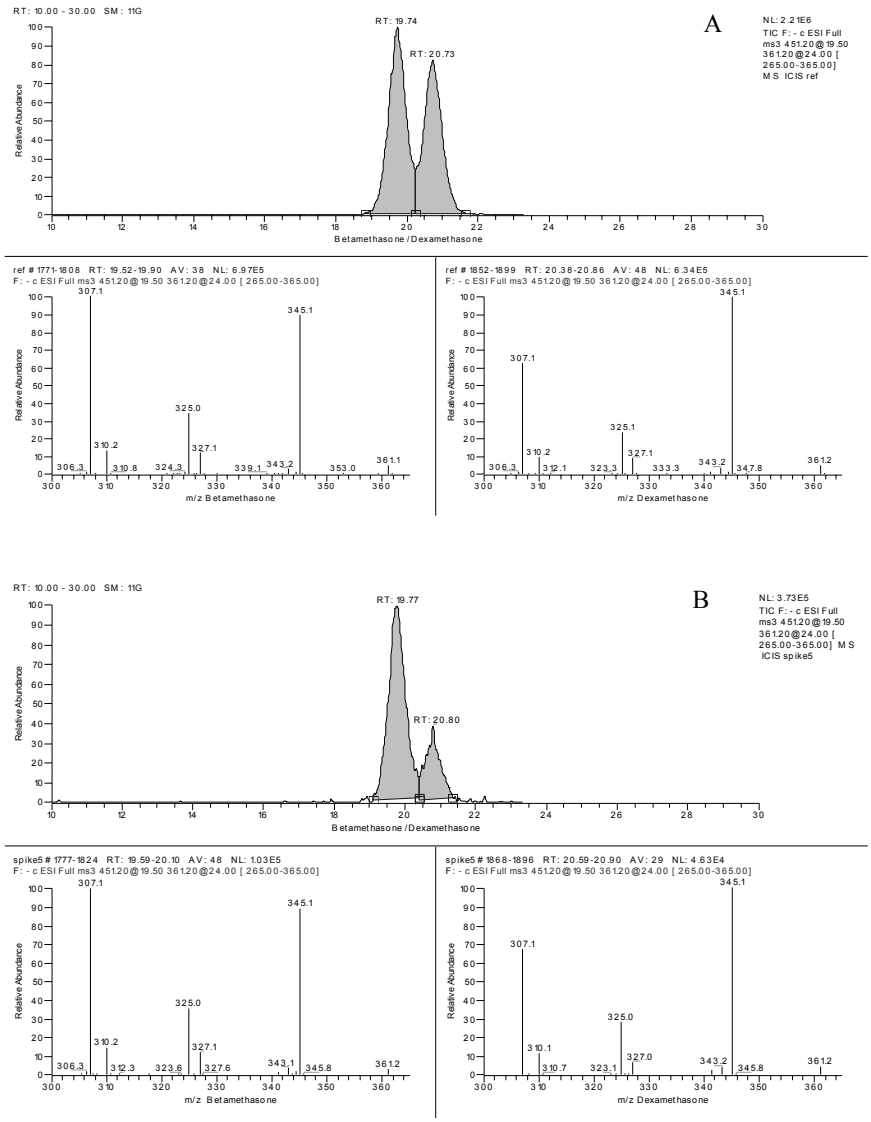
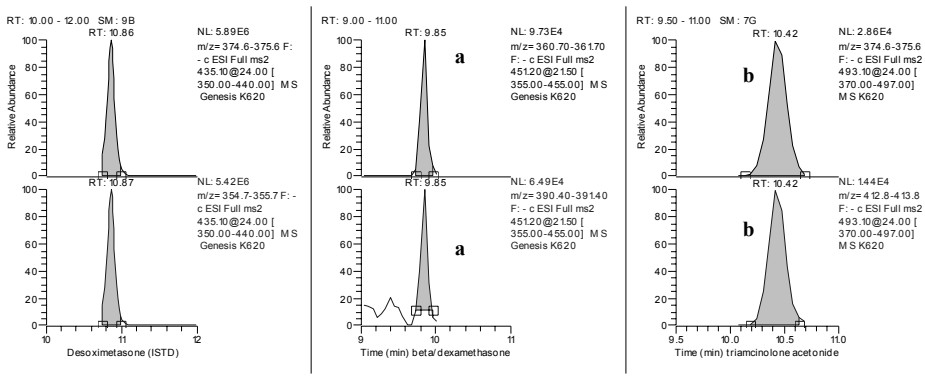


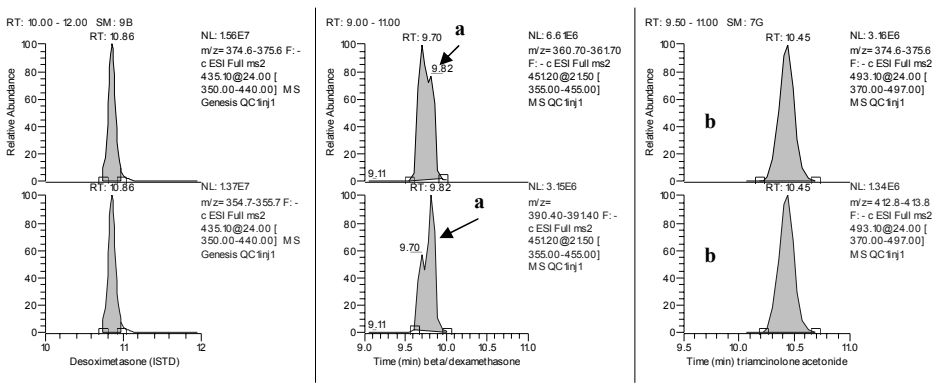
Figure 3: TIC and MS^3 spectra for betamethasone and dexamethasone: A. reference mixture (100 ng/ml); B. Control urine spiked with dexamethasone and betamethasone at 1 and 2 ng/ml, respectively.

Routine application

During the summer of 2002 more than 400 routine samples were analysed using this screening method, twelve samples were found to contain corticosteroids. Two of them contained methylprednisolone, five were found to contain betamethasone, four triamcinolone acetonide and one urine sample contained both dexamethasone and triamcinolone acetonide (Figure 4).



A



B

Figure 4: A: Positive urine sample containing both dexamethasone (a) and triamcinolone acetonide (b). B: Betamethasone, dexamethasone and triamcinolone acetonide spiked in a quality control urine (10 ng/ml).

Conclusion

An LC-ESI-MS^2 screening method for 9 corticosteroids in urine was developed and validated. A method to differentiate between betamethasone and dexamethasone was also validated. This method allowed us to reduce sample analysis costs by avoiding the use of different ELISA kits. The routine application of this screening method demonstrates that corticosteroids can be detected unconjugated in urine.

Acknowledgements

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3. Excretion studies with corticosteroids

Adapted from:

Deventer K, Delbeke FT.

Excretion studies with corticosteroids.

Recent Advances in Doping Analysis (12). Eds: Schänzer W, Geyer H, Gotzmann A, Mareck U. Sport und Buch Strauβ, Cologne, 2004: 37.

Introduction

The therapeutical and also doping use of corticosteroids is widespread since several decades. Their detection in biological matrices has been problematic for a long time. The existing analytical techniques including GC-MS, ELISA or HPLC-UV show poor sensitivity or selectivity.
However since the introduction of liquid chromatography coupled to mass spectrometry (LC-MS) this problem could be solved. LC-MS shows very good sensitivity and selectivity for corticosteroids and as a consequence different papers on their detection in doping analysis by LC-MS have been published [1-4].
Corticosteroids are commercially available in different forms e.g. ointments, inhalers, injectable solutions and pills. Unfortunately no information is available about the detection times after the use of these preparations. Recently, a screening method for the detection of nine commonly used and abused corticosteroids has been validated in our laboratory [5]. This screening method was applied for the detection of betamethasone, triamcinolone acetonide, beclomethasone, budesonide and methylprednisolone following different administration routes.

Excretion studies

All studies were reviewed and approved by the ethical committee of the institution (UZGent, Projects EC/2005-81/sdp and 2003/384). Each volunteer signed a statement of informed consent.

Oral administration

One tablet of the following commercially available drugs was orally taken by one female patient and one male volunteer, aged 57 and 26 years respectively: Medrol® containing 32 mg of methylprednisolone, Pharmacia (Diegem, Belgium) and Celestone® containing 0.5 mg betamethasone, Schering-Plough (Brussels, Belgium). Urine samples were collected at 0h and after 1, 2, 3, 6, 9, 12, 24, 48 and 72h.

Dermatological application

A single dose (0.5 g) of the following commercially available ointments was applied topically between the buttocks by two male volunteers, aged 26 and 34 years respectively: Diprosone® 0.05% (0.643 mg betamethasone dipropionate per gram of ointment) Schering Plough (Brussels) and Delphi® containing 0.1% triamcinolone acetonide, Lederle (Louvain-la-Neuve, Belgium).
Urine samples were collected at 0h and after 1, 2, 3, 6, 9, 12, 24 and 48h.

Administration by inhalation

Two formulations were tested: a single dose of Pulmicort®, Astra-Zeneca (Brussels), containing 0.2 mg of budesonide was administered by inhalation using a turbohaler by a male patient aged 23 and in another experiment one dose of Becotide®, GlaxoSmithKline (Genval, Belgium), containing 0.25 mg of beclomethasone dipropionate was administered by inhaler.
Urine samples were collected at 0h and after 1, 2, 3, 6, 9, 12, 24h.

Parenteral administration

Diprophos®, Schering-Plough (Brussels), containing betamethasone phosphate and betamethasone dipropionate (equivalent to 7 mg betamethasone) was administered intramuscularly to one male patient, aged 34 and intra-articularly to 2 male patients aged 55 and 36, respectively. In another experiment Diprophos® (equivalent to 14 mg betamethasone) was administered intramuscularly to a male patient aged 37.
Kenacort®, Bristol-Myers (Braine l'Alleud, Belgium) containing 40 mg triamcinolone acetonide was injected intra-articularly to a male patient.
Urine samples were collected at 0h and after 1, 2,3, 6, 9, 12 and 24h and then on a daily basis for 20 days.

Sample preparation

The internal standard solution (50 µl desoximetasone, 1 µg/ml) was added to 5 ml of urine, followed by addition of 1 g of potassium carbonate. Five ml diethylether were added and the sample was extracted by rolling for 20 minutes. After centrifugation (1200g) the organic layer was transferred into a new tube and evaporated to dryness under OFN at 40 ºC. The residue was dissolved in 200 µl mobile phase.

A TSP Model P4000 quaternary pump, equipped with a TSP Model AS 3000 autosampler with a 100 µl sample loop and connected to a Finnigan MAT LCQ-Deca® mass spectrometer was used. Separation was performed on a Nucleosil column (Varian, Sint-Katelijne-Waver, Belgium). The mobile phase consisted of 1% acetic acid (solution A) and acetonitrile. Gradient elution at a flow rate of 0.3 ml/min was as follows: 70% A for 5 min, linear gradient to 35% A in 2 min, isocratic for 5 min, followed by 70% A with 8 min equilibration before the next injection. Total run time was 20 min.

Mass spectrometry was performed on a LCQ DECA instrument in ESI negative ionisation mode for all compounds except 16α-hydroxyprednisolone, which was screened for in positive mode. From the MS^2 spectrum two abundant ions were selected for monitoring the presence of a corticosteroid.

Results and discussion

Oral administration

Already one hour after administration methylprednisolone and betamethasone were detected in the urine samples. Methylprednisolone could be detected up to 48h after intake while betamethasone could be detected up to 72 h.

Dermatological application

Betamethasone could not be detected in the administration samples. The administration was repeated by applying a double dose (i.e. 1 g) of ointment but betamethasone was

still not detected.

The dermatological application of Delphi® ointment resulted in the detection of triamcinolone acetonide from 2h until 24h after administration (Figure 1).

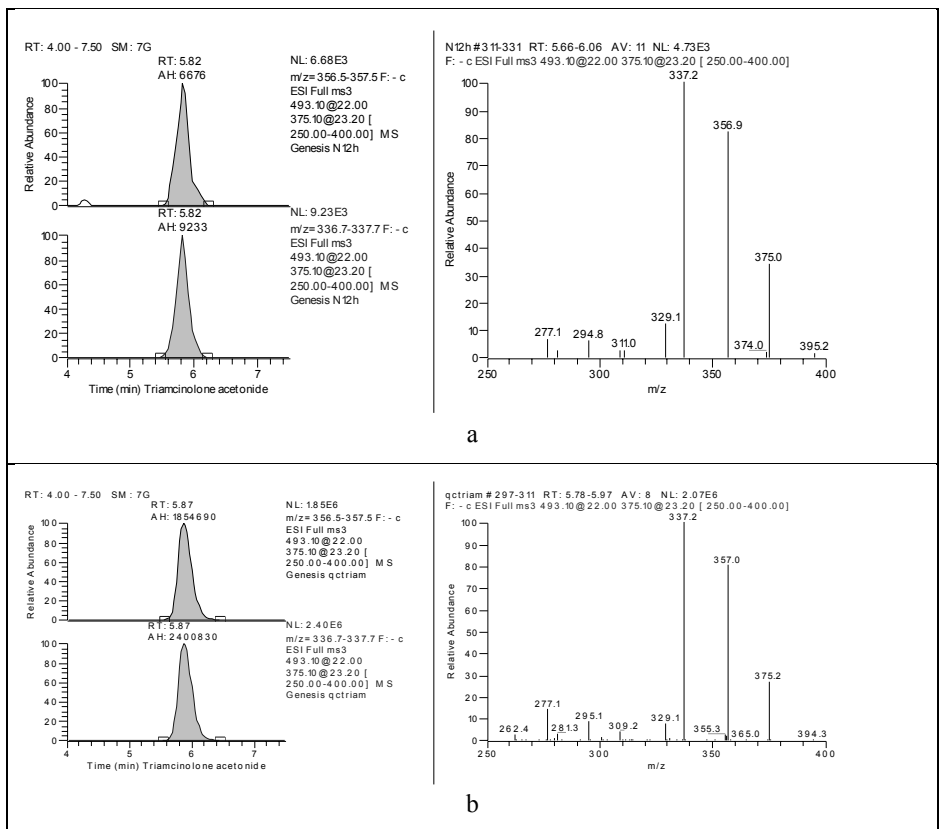

Figure 1: Selected ion chromatograms and MS^3-spectrum for triamcinolone acetonide in a urine sample (a) 12 h after dermatological application of triamcinolone acetonide ointment (0.1 %) and a control urine (b) spiked at 10ng/mL.

Administration by inhalation

Inhaled corticosteroids are mostly used in the treatment of asthma. Budesonide, beclomethasone, fluticasone and flunisolide are most commonly used.

Recently the detection of non conjugated beclomethasone in equine urine and plasma was reported [6]. In this study beclomethasone could not be detected after

administration of a single dose of beclomethasone dipropionate. A possible reason could be the low dose and the fact that the drug and its metabolites are excreted mainly in faeces [7].

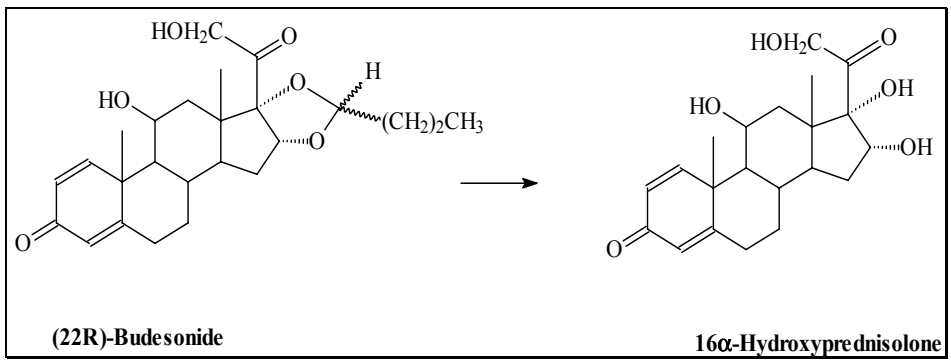

Figure 2: Metabolic pathway of budesonide

As shown in Figure 2 budesonide is metabolised to 16 α–hydroxyprednisolone by cleavage of the 16α, 17α–acetal group. The reaction requires oxidative and hydrolytic enzyme activity [8].

In the urine samples budesonide could be detected for 1h while 16α-hydroxyprednisolone remains detectable up to 9 hours after administration. Ion chromatograms and mass spectra for budesonide and 16α-hydroxyprednisolone after the inhalation of budesonide are presented in Figure 3(a) and (b).

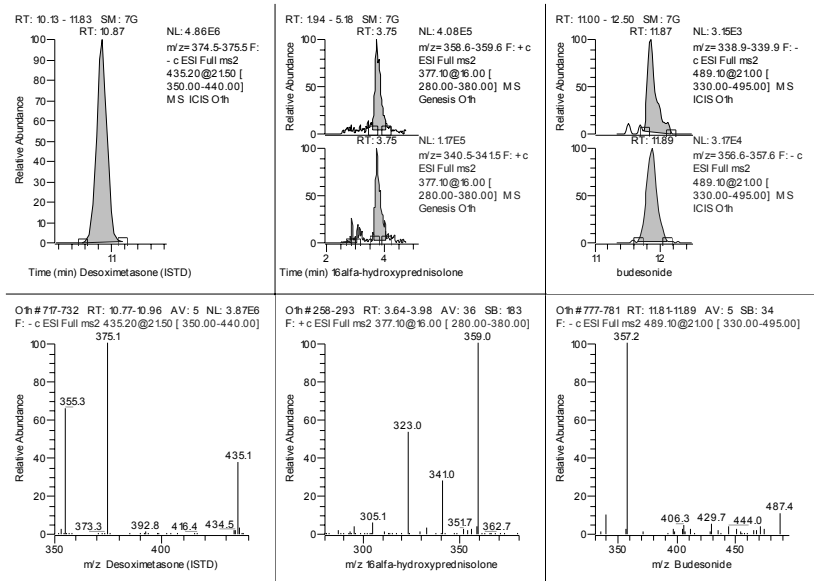

Figure 3(a): Urine sample 1h after inhalation of 200 µg of budesonide

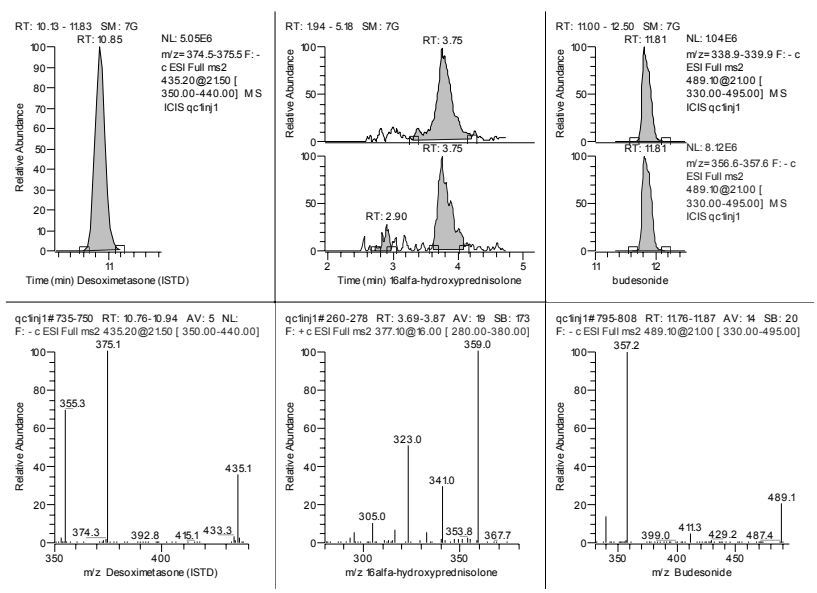

Figure 3(b): Blank urine spiked at 10 ng/ml with 16α-hydroxyprednisolone and budesonide

Parenteral administration

Corticosteroids can be detected for a long period after injection (Table 1).
After intra-articular injection of Kenacort®, triamcinolone acetonide could be detected up to 12 days. Intra-articular injected betamethasone could be detected from 1h until 14 days post administration. In one excretion study with intramuscular injected betamethasone the detection time was underestimated (urine collection was ended after 20 days while betamethasone was still detectable).

Table 1: Detection times of some corticosteroids after parenteral administration

Commercial name	Active Compound	Amount	Injection	Detection time
Diprophos®	Betamethasone	7 mg	IM	12 days
Diprophos®	Betamethasone	14 mg	IM	>20 days*
Diprophos®	Betamethasone	7 mg	IA	14 days
Diprophos®	Betamethasone	7 mg	IA	14 days
Kenacort®	Triamcinolone acetonide	40 mg	IA	12 days

*urine collection was ended after 20 days
IM: intramuscular, IA: intra-articular

Conclusions

The major goal of this study was to establish urinary detection times for several corticosteroids after different administration routes.
For triamcinolone acetonide and budesonide a single dose of 0.5 mg (topically) or 0.2 mg (inhaled) could be detected in urine up to 24 hours and 9 hours, respectively.
Long detection times were observed for the injected corticosteroids, up to 20 days for betamethasone.
Including the 16α-hydroxyprednisolone metabolite in the screening method seemed to be a successful approach for the detection of inhaled budesonide.
Inhaled beclomethasone and topical applied betamethasone could not be detected in the

urine samples.

The results presented here were carried out with a heterogeneous group of volunteers taking each a different preparation. Hence, further research will be performed with the administration of one preparation to multiple volunteers.

Acknowledgment

The authors are grateful to the Belgian National Lottery for the purchase of the LCQ-DECA® instrument and the Ministry of the Flemish Community for a grant.

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Mass Spectrom. 2003; **38:** 823.

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4. Detection of budesonide in human urine after inhalation

Adapted from :

Deventer K, Mikulčíková P, Van Hoecke H, Van Eenoo P, Delbeke FT.
Detection of budesonide in human urine after inhalation by liquid chromatography-mass spectrometry.
J. Pharm. Biomed. Anal. 2006; **42**: 474.

Abstract

Budesonide, a corticosteroid frequently used in the treatment of asthma, is most often administered via inhalation. Its use in sports is allowed when medically necessary. A fast, sensitive and accurate LC-MS method was developed and validated for the quantification of budesonide and its major metabolite 16α–hydroxyprednisolone in urine samples after inhalation of a metered dose (Pulmicort-Turbohaler®200). Sample preparation consists of an alkaline liquid-liquid extraction with ethyl acetate. Analysis was performed using liquid chromatography-tandem mass spectrometry with ESI.

The method was linear in the range 5-100 ng/ml and 0.5-10 ng/ml for 16α–hydroxyprednisolone and budesonide, respectively. The limits of quantification were 5 ng/ml for 16α–hydroxyprednisolone and 0.5 ng/ml for budesonide.

The inaccuracy ranged from 2.2 to 3.5 % for 16α–hydroxyprednisolone and from 0.8 to 16.4 % for budesonide. After administration of 200 µg of budesonide to 5 healthy volunteers budesonide could not be detected in any urine sample whereas 16α–hydroxyprednisolone was detectable up to 12 hours post administration.

Introduction

Corticosteroids are very powerful anti-inflammatory agents used for the treatment of inflammatory diseases such as asthma. They can cause euphoria [1] and they alleviate pain in general, allowing athletes to perform while they are actually injured. Hence corticosteroids appear on the prohibited list of substances issued by WADA [2]. Athletes use budesonide (Figure 1) mainly for the treatment of excercise induced asthma and it is a frequently administered corticosteroid by inhalation. Budesonide is rapidly metabolised to different metabolites of which 16α-hydroxyprednisolone (Figure 1) is the major one in man [3]. Hence this metabolite is the primary target compound for the detection of budesonide in doping analysis [4]. Despite structural similarities between budesonide and desonide regarding the acetal moiety at the 16 and 17 position, 16α-hydroxyprednisolone was not detected as a metabolite of desonide [5].

Although different pharmacokinetic studies [3,5,6] have been published, few data on the detection of 16α -hydroxyprednisolone and budesonide related to doping analysis is available [7]. The aim of the current study was to determine budesonide and 16α–hydroxyprednisolone in urine after inhalation of a single dose of budesonide using a Pulmicort®-Turbohaler®200.

Experimental

Chemicals and reagents

Betamethasone and budesonide were a gift from Glaxo-Wellcome (Greenford , United Kingdom), 16α–hydroxyprednisolone was a gift from Astra-Zeneca (Lund, Sweden). Analytical grade potassium carbonate, sodium hydrogen carbonate and acetic acid were from Merck (Darmstadt, Germany) and ethyl acetate from Acros (Geel, Belgium). HPLC grade water was obtained from Fischer (Loughborough, United Kingdom) and acetonitrile from Biosolve (Valkenswaard, The Netherlands). Gases used in mass spectrometry were helium (Alphagaz-grade) and nitrogen (LASAL2001-grade) both purchased from Air Liquide (Desteldonk, Belgium).

Excretion study

The study was performed with five healthy male volunteers aged 24, 26, 29, 33 and 37. The study protocol was reviewed and approved by the ethical committee of the institution (UZGent, Project 2005-160). Each volunteer signed a statement of informed consent and inhaled 200 µg of budesonide during 5 seconds using a Pulmicort® - Turbohaler®200 (AstraZeneca, Brussels, Belgium). Urine samples were collected before (0h) and quantitatively 1, 2, 3, 6, 9 and 12 h after administration. Additional samples were taken 24 and 48 h after inhalation. All urine samples were either analyzed directly or stored at -20°C awaiting analysis. All samples were analysed in duplicate.
Sample treatment

An internal standard (IS) solution (50 µl betamethasone, 1 µg/ml) was added to 5 ml urine, followed by the addition of 200 mg of a solid buffer containing sodium hydrogen carbonate and potassium carbonate (2:1 w/w) to adjust the pH to 9.2. Liquid-liquid extraction was performed by rolling for 10 min with 4 ml ethyl acetate. After centrifugation (1200g) the organic layer was transferred into a new tube and evaporated until dry under OFN at 40 ºC. The remaining residue was dissolved in 200 µl of the initial mobile phase, 50 µl was injected into the HPLC-system.

Instrument parameters

Separation of the compounds was performed on an Omnispher C18 column 50 x 3 mm, 3 µm protected with a guard column 10 x 2 mm, both from Varian (Sint-Katelijne-Waver, Belgium) using a Surveyor LC-pump and a Surveyor autosampler both from Thermo (San Jose, USA). The column temperature was kept at 35 °C.
The mobile phase consisted of an aqueous solution of 1 % acetic acid (A) and acetonitrile (B). Gradient elution at a flow rate of 0.4 ml/min was as follows: 90% A for 0.25 min, followed by a linear decrease in A to 10% in 1.25 min, isocratic for 4.5 min, followed by an increase in 0.5 min to 90% A which was maintained for 4.5 min before the next injection. The total run time of the method was 11 min.
Detection was carried out using a Thermofinnigan LCQ-Deca® XP Plus mass spectrometer (Thermo, San Jose, California, USA) using ESI in positive mode. The ion source voltage was 5000 V and the sheath gas and the auxilliary gas flow rate were set at 80 and 10 units, respectively. The capillary voltage was 20 V.
Full scan tandem mass spectrometry was applied for all compounds. The isolation width was set at 3.0. Activation time and Q value were set arbitrarly at 30 ms and 0.250. The collision energy was set at 25 % for all compounds.

Validation

A five-point calibration curve was generated by spiking blank urine with 16α–hydroxyprednisolone and budesonide in triplicate at 5, 10, 20, 50, 100 ng/ml and

0.5, 1, 2, 5, 10 ng/ml, respectively. Averages were used to construct the calibration curve.

The area ratios of the product ions of budesonide (m/z 413) and 16α–hydroxyprednisolone (sum of m/z 323, 341, 359) and the product ion of the internal standard (m/z 373) were plotted versus the concentration.

Precision and inaccuracy of the method were tested at three levels (0.5, 2, 10 for budesonide and 5, 20, 100 ng/ml for 16α–hydroxyprednisolone). Precision was assessed as the percentage RSD of both repeatability (within-day) and reproducibility (between-day and different analysts) for a selected compound and level. Maximum allowed tolerances for reproducibility and repeatability can be calculated from the Horowitz-equation $RSD_{max} = 2^{(1-0.5\log C)}$ (C = concentration (μg/ml)*10^{-6}). The maximum allowed tolerances for repeatability and reproducibility are $2/3RSD_{max}$ and RSD_{max}, respectively [8].

Inaccuracy was defined as the difference between the calculated amount and the specified amount for the selected compound and expressed as a percentage [9].

The LOQ of the method was defined as the lowest concentration where acceptable reproducibility and inaccuracy could be guaranteed. The LOD was defined arbitrarily as ½ LOQ.

Selectivity was tested by analysing several structurally related and other routinely screened doping agents, including corticosteroids and anabolic steroids. Concentrations in these mixtures were 1 μg/ml.

Specificity was tested by analysing ten blank urines.

In each batch of excretion urine samples, a blank urine sample, a system blank (aqua bidest) and a quality control sample (spiked at 2 and 20 ng/ml were analysed concurrently for respectively budesonide and 16α–hydroxyprednisolone).

Extraction recovery

Ethyl acetate, dichloromethane and diethylether, extraction solvents routinely used in our laboratory, were evaluated. Therefore negative urine samples (n= 6) were spiked with 16α–hydroxyprednisolone and budesonide at 5 ng/ml and extracted together with non spiked negative urine samples (*n*=6). The extracts of the non spiked urine samples

were then spiked at 5 ng/ml simulating a 100% recovery. Both sets of samples were evaporated and analysed as described. The obtained peak areas of the two sets of samples were compared to evaluate recovery.

Results and Discussion

Method development

The described HPLC method is an adaptation of a previously published screening method for corticosteroids [4]. By reducing the column length from 100 to 50 mm a decrease in the analysis time by a factor of 2 was obtained.

Under the chromatographic conditions described, all compounds eluted as sharp peaks within a short time range. Retention times were 5.01 min, 5.31 min and 5.71 min for 16α–hydroxyprednisolone, betamethasone and budesonide, respectively.

Diagnostic ions were determined by flow injection analysis. For each compound a solution of 5 µg/ml was infused at a flow rate of 10 µl/min. In full scan MS, abundant protonated molecular ions were observed for all compounds. In the full scan MS^2 spectrum of budesonide (molecular weight 430) one intense product ion was observed at m/z 413 $[MH\text{-}H_2O]^+$ and several minor product ions at m/z 395, 341 and 323 (Figure 1). Despite the low specificity of product ions generated by the loss of water [10] the product ion (m/z 413) was used as diagnostic ion for the detection of budesonide. Unlike budesonide, 16α–hydroxyprednisolone (molecular weight 376) exhibited intensive fragmentation and several intense product ions were observed, namely m/z 359, 341 and 323 (successive losses of H_2O). To avoid loss of sensitivity due to the intensive fragmentation of this compound the sum of these three ions was used for quantification. For the internal standard betamethasone (molecular weight 392) the product ion m/z 373 $[MH\text{-}HF]^+$ was used.

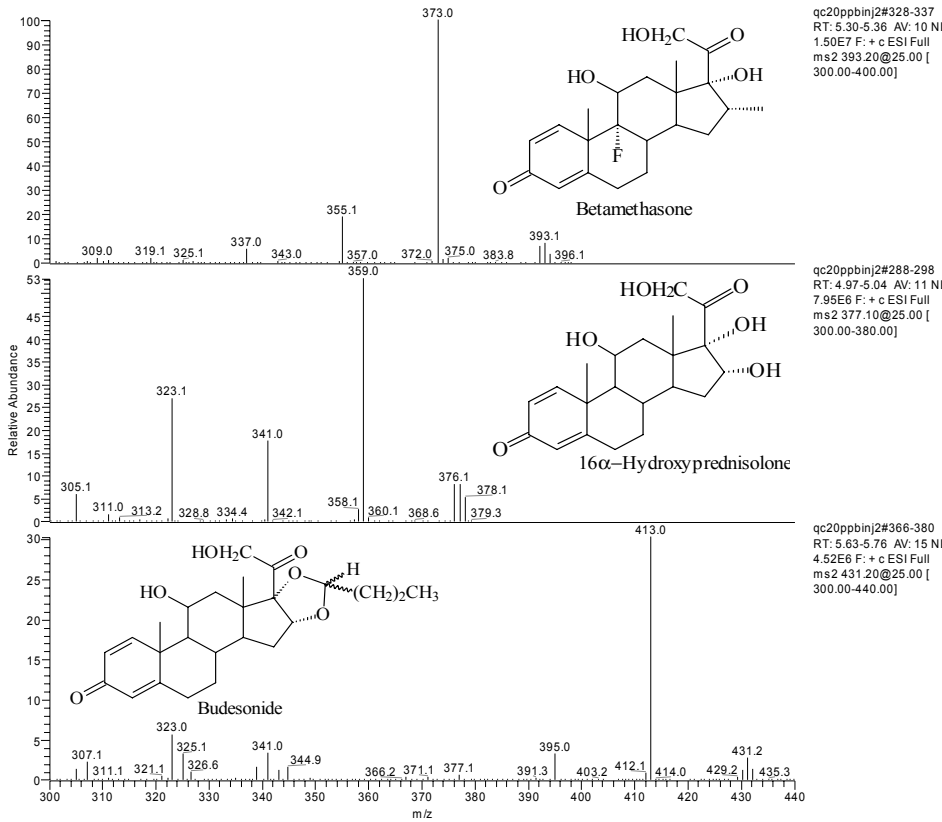

Figure 1: Product ion mass spectra of betamethasone (IS), 16α–hydroxyprednisolone and budesonide.

Extraction recovery

Recoveries for the different compounds are given in Table 1. For budesonide relatively small differences were observed between the different solvents. However great differences were observed for 16α–hydroxyprednisolone. Both dichloromethane and diethylether showed poor recovery for 16α–hydroxyprednisolone whereas ethyl acetate gave far better recoveries (Table 1). As a consequence ethyl acetate was preferred as extraction solvent.

Table 1: Extration recoveries for 16α-hydroxyprednisolone and budesonide[a].

Substance	Extraction recovery (%) (*n*=6)		
	dichloromethane	diethylether	ethyl acetate
16α-Hydroxyprednisolone	12.1 ± 1.2	9.8 ± 1.1	59.5 ± 2.7
Budesonide	85.4 ± 1.2	91.9 ± 0.56	87.7 ± 1.1

[a] values are presented as mean ± standard deviation (*n*=6), concentrations 5 ng/ml.

Method validation

Using a least square fit, good linearity ($r^2 \geq 0.998$) was observed for 16α–hydroxyprednisolone and budesonide in the range 5 - 100 ng/ml and 0.5-10 ng/ml, respectively. None of the calibration curves was forced through the origin and for the regression calculation a weighing factor of 1/x was used for all data points. The results for precision and inaccuracy are summarised in Table 2.

Table 2: Inaccuracy, repeatability, reproducibility and tolerance limits of the LC-MS method at three concentrations including the lowest point of the calibration curve for 16α-hydroxyprednisolone and budesonide.

Substance	Conc. [ng/ml]	Inaccuracy [%] *n=18*	Repeatability [%] *n=6*	Reproducibility [%] *n=18*	RSD_{max} [%]	2/3 RSD_{max} [%]
16α-Hydroxy-prednisolone	5	3.5	7.1	8.5	32	21
	20	1.3	10.4	10.1	25	19
	100	2.2	3.4	5.8	23	15
Budesonide	0.5	16.4	5.2	4.9	50	33
	2	-3.2	9.5	7.5	41	27
	10	0.8	1.6	6.6	32	21

As shown in Table 2, allowed tolerances were not exceeded neither for repeatability nor reproducibility. Deviation of the mean measured concentration from the theoretical concentration (inaccuracy) for all compounds was below the acceptable threshold of 15% and 20 % [9] for all levels in the calibration curve.
Regarding the selectivity, interference from other monitored doping agents could not be found. In addition analysis of 10 different blank control urine samples did not result in the detection of interfering substances, proving the specificity of the method.
The LOQ of the method was 5 ng/ml for 16α–hydroxyprednisolone and 0.5 ng/ml for budesonide. The LOD was 2.5 ng/ml and 0.25 ng/ml for 16α–hydroxyprednisolone and budesonide, respectively.

Administration urine samples

Budesonide could not be detected in any of the post administration urine samples in accordance with the intensive and fast metabolism [5].
Chromatograms of a blank urine, a quality control urine and an administration urine sample are presented in Figure 2.
The urinary excretion profiles for 16α–hydroxyprednisolone are shown in Figure 3. 16α–hydroxyprednisolone could already be detected 1 hour after inhalation, except in subject 1. Maximum urinary concentrations of 16α–hydroxyprednisolone were obtained 2-3 h after inhalation, except in subject 1 (T_{max} 6 h).
The maximum urinary concentrations ranged between 10 and 79 ng/ml. These large differences are at least partially caused by differences in urine volumes. In 4 out of 5 subjects 16α–hydroxyprednisolone was detectable until 12 hours post administration.
Cumulative excretion data (Figure 3) indicate that between 3 and 13% of the administered dose is excreted as 16α–hydroxyprednisolone. These variations could be due to a poor use of the turbohaler in accordance with studies reporting a 37 % recovery of budesonide from the inhalation device after application [11]. However, other reasons including poor resorption cannot be excluded.

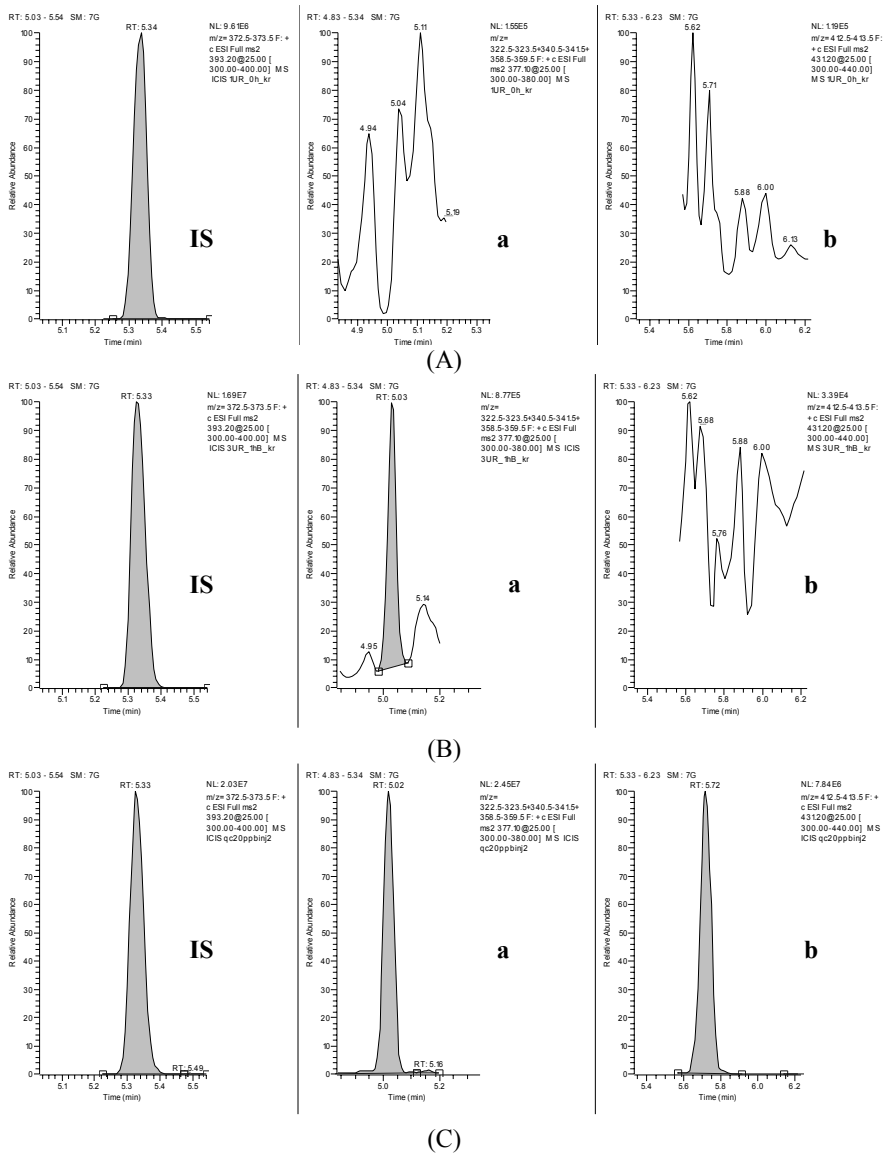

Figure 2: Ion chromatograms obtained from blank urine (A), urine sample 1 hour after inhalation (B), and a control urine (C) spiked at 20 and 2 ng/ml with 16α–hydroxyprednisolone (a) and budesonide (b), respectively.

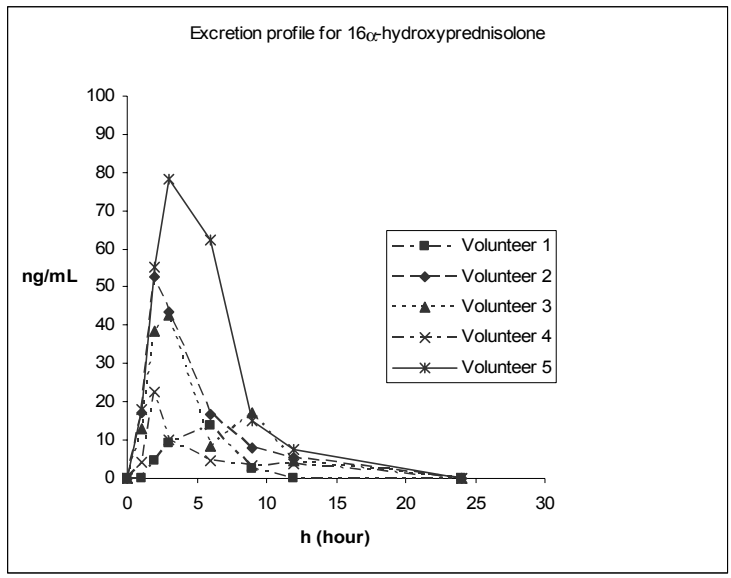

Figure 3(A)

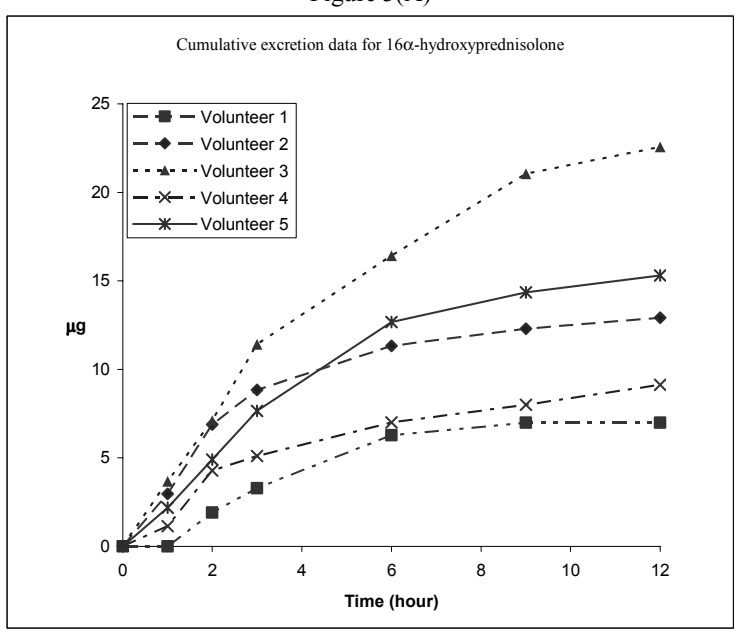

Figure 3(B)

Figure 3: Urinary concentrations (A) and cumulative excretion (B) of 16α-hydroxyprednisolone after the inhalation of a single dose of budesonide (200 µg).

Conclusions

A quantitative LC-MS method has been developed and validated for the detection of 16α–hydroxyprednisolone and budesonide in urine after inhalation of 200 µg budesonide.
Administration studies showed that 16α–hydroxyprednisolone is the target compound in urine for the detection of budesonide application by inhalation.

Acknowledgements

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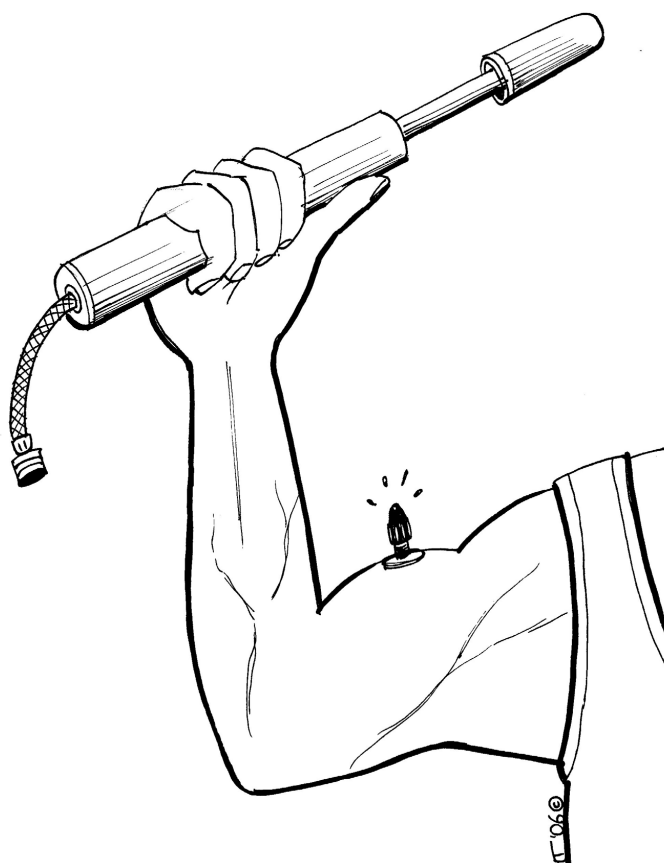
Chapter IV: Anabolic Steroids



1. Introduction

Therapeutic use

Anabolic steroids are drugs derived from the male hormone testosterone. This steroid was first synthesised in 1935 by Butenandt et al. [1]. Similar to corticosteroids, anabolic steroids are also derived from cholesterol. Testosterone is predominantly synthesised in the testes and in small amounts by the ovaries in females as well in the adrenal cortex in both sexes [2].

Anabolic steroids are therapeutically used to treat hypogonadism, delayed puberty, osteoporosis and some types of impotence [2]. They are also prescribed to treat body wasting in patients with AIDS and other diseases which result in loss of lean muscle mass. Some selected anabolic steroids are presented in Figure 1.

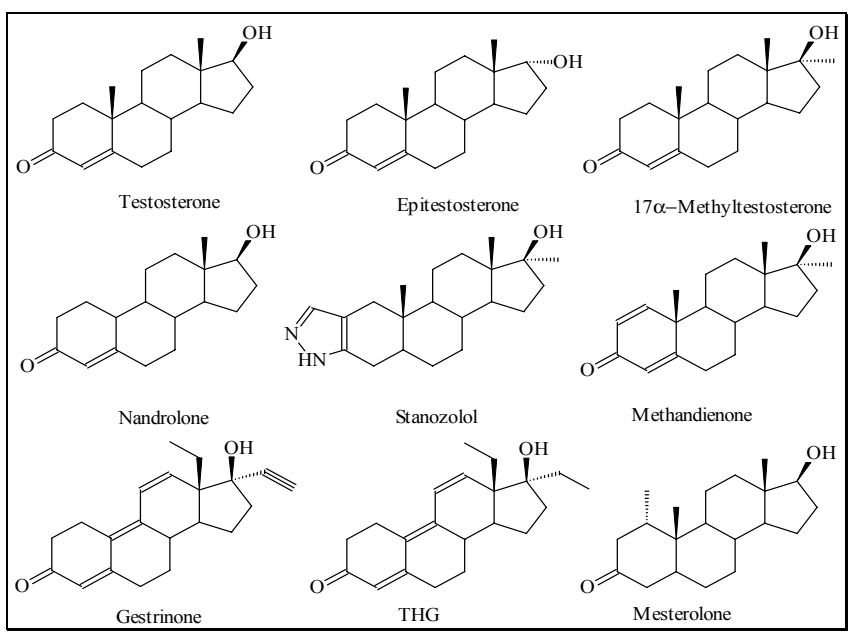


Figure 1: Molecular structures of some selected anabolic steroids.

Efforts have been directed towards the production of synthetic analogues of testosterone, with retention of the protein anabolic action of testosterone and are relatively exempted of androgenecity, an undesired side effect of anabolic steroids.

Doping use

Anabolic steroids are abused by athletes to increase muscle mass and affect performance.
In bodybuilding anabolic steroids are used to improve physical appearance.
Anabolic steroids are on the list of prohibited substances published by WADA [4].
Despite the severe side effects of anabolic steroids (testicular cancer, sterility, inhibition of ovulation) some steroid abusers take several decades higher doses than therapeutically recommended [3]. Allthough a wide variety of doping substances are nowadays available, anabolic steroids remain very popular. More than 40 % of all reported substances in 2005 were anabolic steroids [5]. Worldwide media attention focussed on anabolic steroids in 1988, when Ben Johnson tested positive for stanozolol and in 2003 with the outbreak of the THG-scandal.

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2. Screening for anabolic steroids

Adapted from:

Deventer K, Van Eenoo P, Delbeke FT

Screening for anabolic steroids in doping analysis by liquid chromatography-electrospray/ion trap mass spectrometry.

Biomed. Chromatogr. 2006; **20**: 429-433.

Abstract

A fast and selective LC-MS^2 method for the screening of 4 anabolic steroids in human urine has been developed and validated. Liquid/liquid extraction with diethylether was applied after enzymatic hydrolysis. Analysis was performed on an ion trap instrument equipped with electrospray ionisation. MS^2 was applied for all compounds. The analytical run time was 11 minutes. The LOD for all compounds varied between 1 and 10 ng/ml. Left over routine samples, which were declared positive by GC-MS for the presence of 3'hydroxystanozolol, were assessed with the described method.

Introduction

The detection of anabolic steroids and/or their metabolites by GC-MS has been extensively described [1-4]. Few methods using LC-MS have been described in the field of human doping analysis [5].

In veterinary residue analysis however LC-MS has been succesfully applied for the detection of anabolic agents, in particular stanozolol and its metabolites [6].

In 2003 tetrahydrogestrinone was discovered as a designer anabolic steroid and its detection was investigated both with GC-MS and LC-MS [7]. Unfortunately, this compound could not be detected using the existing GC-MS screening methods for anabolic steroids and Catlin et al. suggested LC-MS as the prefered detection technique [7]. Hence, a routine screening method using LC-APCI-MS^2 was developed in our laboratory. In this study, this method was further optimised for the detection of another 3 anabolic steroids including gestrinone, 3'hydroxystanozolol (major metabolite of stanozolol) and 17α-trenbolone.

Experimental

Chemicals and reagents

17TREN was purchased from Roussel-UCLAF (Neuville-sur-Saone, France), and 3OHSTAN from National Measurement Institute (Sydney, Australia). THG was a kind gift of the UCLA Olympic Analytical Laboratory of Los Angeles (USA). GES and 16OHSTAN were kind gifts from the Cologne (Germany) doping control laboratory.
The internal standard fluoxymesterone was obtained from Pfizer (Puurs, Belgium) and the β-glucuronidase preparation (type E. Coli K 12) was purchased from Roche (Mannheim, Germany).
Analytical grade potassium carbonate, sodium hydrogen carbonate, formic acid and diethylether were from Merck (Darmstadt, Germany), HPLC grade methanol was from Acros (Geel, Belgium) and HPLC grade water from Fischer (Loughborough, UK).

Sample preparation

To five ml of urine fortified with the internal standard solution (50 µl fluoxymesterone, 10 µg/ml), 1 ml of a phosphate buffer (pH 7) and 50 µl of enzyme solution were added. The sample was hydrolysed for 2.5h at 56 °C. After cooling to room temperature, 200 mg of a solid buffer containing sodium hydrogen carbonate and potassium carbonate (2:1 w/w) was added to adjust the pH to 9.2. Liquid-liquid extraction was performed by rolling for 10 min with 5 ml diethylether. After centrifugation (1200g) the organic layer was transferred into a new tube and evaporated until dry under OFN at 40 ºC. The remaining residue was dissolved in 200 µl mobile phase.

Validation

The validation was carried out following Eurachem validation guidelines [8].
Ten urines were spiked at 6 different levels with the four steroids. Final concentrations were 10, 4, 2, 1, 0.5 and 0.1 ng/ml. The samples were extracted as described above.

The detection limit was defined as the lowest level at which a compound could be identified in all 10 urines with a S/N ratio greater than 3 and a retention time (RT) that differs not more then 0.2 min from the RT in the reference mixture.
Selectivity was tested by analysing several other doping agents including other anabolic agents, beta-blockers, narcotics, diuretics, corticosteroids and stimulants using the described method. Specificity was tested during the validation procedure. Ten blank urines were extracted and analysed as described above.

Apparatus

A Surveyor quaternary pump and a Surveyor autosampler (both from Thermo, San Jose, USA) were used. The autosampler was equipped with a 100 µl sample loop.
Detection was by a Thermo LCQ-Deca® XP PLUS-mass spectrometer (Thermo, San Jose, USA).

LC-parameters

An Omnispher C18 column 50 x 3 mm, 3 µm protected with a guard column 10 x 2 mm, both from Varian (Sint-Katelijne-Waver, Belgium) was used for chromatographic separations.
The mobile phase consisted of a mixture of 0.1% formic acid and methanol (40/60). Isocratic elution at a flow rate of 0.3 ml/min was performed in an 11 min run.
A volume of 50 microliter was injected.

MS-parameters

Ionisation of analytes was carried out using ESI. The capillary temperature was maintained at 300 ºC, the ion source voltage at 5000 V and the sheath gas (nitrogen) was set at 70 units. The capillary voltage was 20 V. For all compounds full scan tandem mass spectrometry was applied. The isolation width was set at 2.0. Activation time and Q value were arbitrarly set at 30 ms and 0.250, respectively.

Routine application

3OHSTAN is mainly excreted in the conjugated fraction and hence a hydrolysis step is recommended for its detection [9]. Nevertheless non conjugated 3OHSTAN is also present in human urine due to the instability of the glucuronide [9]. Four left over routine doping samples, in which 3OHSTAN was confirmed by our routine GC-MS method were extracted with and without the hydrolysis step and analysed using the described method.

Results and discussion

Method development

Both ESI and APCI can be used for the detection of anabolic agents [10]. Hence both interfaces were tested. Therefore a solution of 5 µg/ml was directly infused into the mass spectrometer operated in positive ionisation. Protonated molecular ions were observed for all compounds with both interfaces. For THG, GES and 17TREN a better signal to noise ratio was observed when APCI was applied. Unfortunately undesired loss of H_2O was observed for these compounds. This non-specific fragmentation could not be decreased by lowering the temperature of the transfer capillary and vaporizer. With ESI this fragmentation was not observed. The poorest result in APCI was observed for 3OHSTAN without loss of H_2O.

Using ESI, the signal for 3OHSTAN was four times better then with APCI. Although the signal of 3OHSTAN was the poorest of the 4 compounds, it was possible to achieve detection levels compliant with the MRPL set by WADA [11] using ESI. Similar compliance was not possible in APCI.

In LC-MS the mobile phase composition can also influence the ionisation proces [12]. By using formic acid instead of acetic acid the signal for 3OHSTAN was improved by a factor of 2 [6], so the former was used. Because acetonitrile and MeOH are most commonly used as organic modifier in liquid chromatography the influence on the signal of both solvents was tested as well. No difference was observed between the two solvents. However MeOH was prefered as it allowed the compounds of interest to better

separate from earlier eluting matrix interferences. Because tandem mass spectrometry often results in improved sensitivity this technique was applied for all compounds. For GES, THG and 17TREN two abundant ions were selected from the MS^2 spectrum. To obtain maximum sensitivity from the complex MS^2 spectrum of 3OHSTAN the sum of 4 abundant ions was used for identification. Where possible, product ions resulting from loss of water were not selected as diagnostic ion as these fragments are highly unspecific [13,14]. Diagnostic ions are presented in Table 1.

Table 1: Limit of Detection (LOD), retention time (RT) and diagnostic ions.

Substance	RT (min)	MW	PI	DI	CE	LOD (ng/ml)	MRPL (ng/ml)
THG	9.39	312	313	239, 241	30	1	10
Gestrinone	4.72	308	309	239, 241	32	1	10
17α-trenbolone	3.86	270	271	243, 271	47	10	10
3'hydroxystanozolol	3.91	344	345	121, 173, 219, 229	43.5	2	2
Fluoxymesterone (IS)	3.83	376	377	299, 281	30	-	-

MW: molecular weight, CE: collision energy, PI: precursor ion, DI: Diagnostic ions

Validation

All compounds could be detected at least at the MRPL. The detection limits are presented in Table 1. Ion chromatograms obtained after analysis of a control urine are given in Figure 1. The described method seems to be very selective as no interferences were detected when other doping products including other anabolic agents, beta-blockers, narcotics, diuretics, corticosteroids and stimulants were analysed. Specificity was satisfactory as no interfering substances at the appropriate retention times were found when 10 blank urines were analysed.

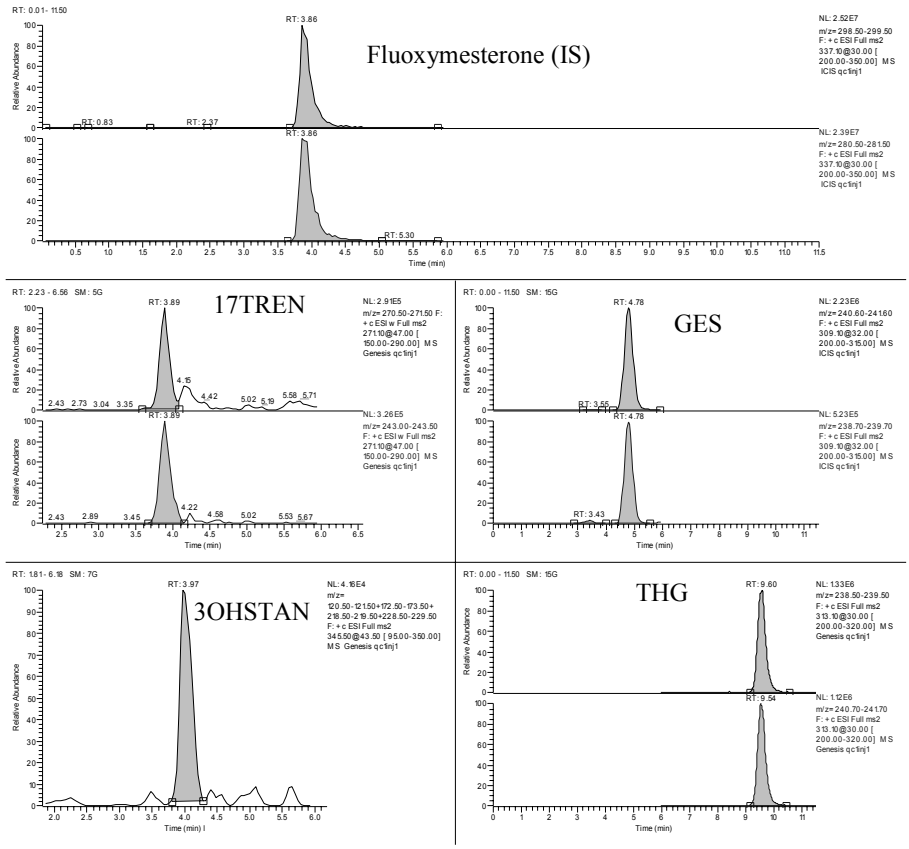

Figure 1: Quality control urine spiked with GES, THG, 17TREN (10 ng/ml) and 3OHSTAN (2 ng/ml).

Routine application

As expected 3OHSTAN could be detected unconjugated in all 4 samples. However, after hydrolysis of the samples the observed signal was at least 50 times higher. In the hydrolysed samples another intense peak was observed showing a similar complex mass spectrum as 3OHSTAN. This peak was assigned to 16OHSTAN after comparing spectra and retention time with a standard solution (Figure 2).

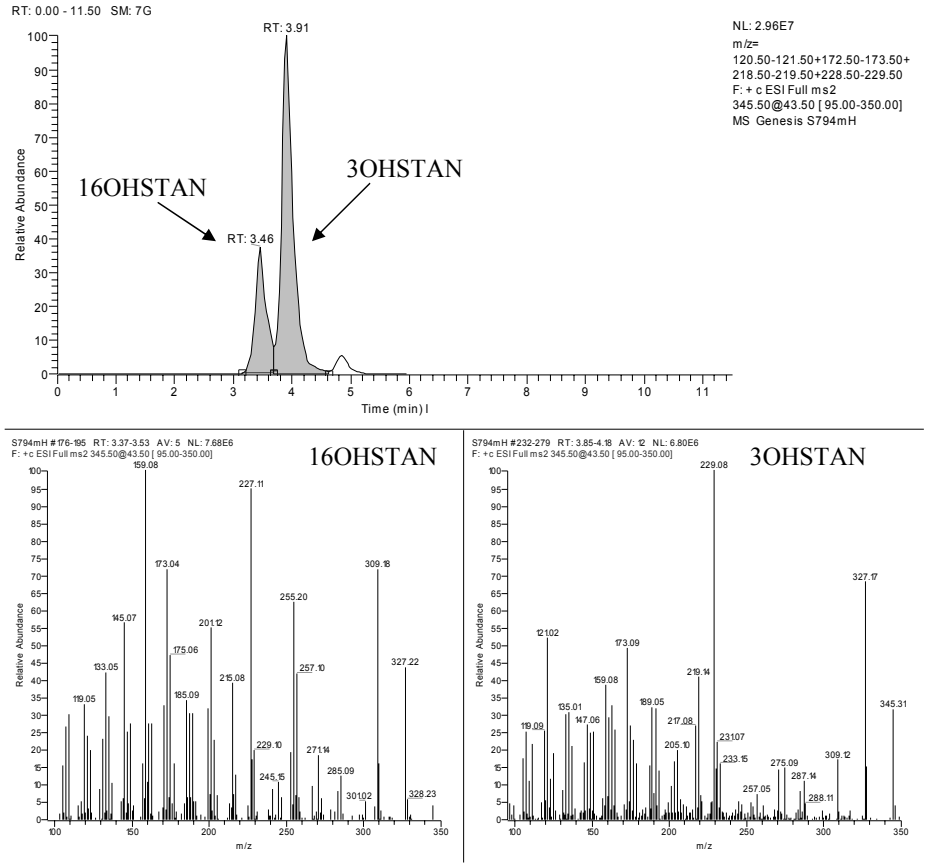

Figure 2: Selected ion chromatograms and mass spectra from a positive urine sample after hydrolysis containing 3'hydroxystanozolol and 16 β-hydroxystanozolol.

Conclusions

A screening method for 4 anabolic agents based on LC-ESI-MS^2 using ion trap technology has been developed and validated. The method seemed to be very sensitive and the detection limits varied between 1 and 10 ng/ml. The method was succesfully applied for the detection of 3'hydroxystanozolol in samples collected for doping analysis.

Acknowledgements

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3. Quantitative analysis of androst-4-ene-3,6,17-trione and metabolites in human urine after the administration of a food supplement

Adapted from:

K. Deventer, P. Van Eenoo, P. Mikulčíková, W. Van Thuyne and F.T. Delbeke
Quantitative analysis of androst-4-ene-3,6,17-trione and metabolites in human urine after the administration of a food supplement by liquid chromatography-ion trap mass spectrometry.
J. Chromatogr. B. 2005; **828**: 21.

Abstract

6-OXO®, a nutritional supplement commercially available on the internet, is sold as an aromatase-inhibitor and contains androst-4-ene-3,6,17-trione as active ingredient. This anabolic steroid is a prohibited substance in sports. A fast, sensitive and accurate LC-MS method was developed and validated for the quantification of androst-4-ene-3,6,17-trione and its metabolites in urine. The method consists of a liquid-liquid extraction step with diethylether after enzymatic hydrolysis, followed by separation on a reversed phase column. Ionisation of the analytes is carried out using atmospheric pressure chemical ionisation. The limit of quantification of the method was 5 ng/ml for all compounds. The inaccuracy ranged from 1.3 to 14.8 % for androst-4-ene-3,6,17-trione, 1.6 to 9.4 % for androst-4-ene-6α-ol-3,17-dione and 3.2 to 4.1 % for androst-4-ene-6α,17β-diol-3-one in the range 5-1000 ng/ml. Using this method androst-4-ene-6α-ol-3,17-dione was identified as the major urinary metabolite, and androst-4-ene-6α,17β-diol-3-one as a minor metabolite. While the parent compound is predominantly excreted in conjugated form, both metabolites are solely excreted as conjugates.

Introduction

Anabolic steroids are widely used by athletes to increase muscle mass [1]. In most cases anabolic steroids are intensively metabolised [2] and the elucidation of the metabolism of an anabolic steroid is necessary to detect its misuse.

During the last decade a number of non-registered anabolic steroids, so-called prohormones, have become available in the United States as nutritional supplements [3]. The term “prohormone” is used because initially these substances were precursors of testosterone or nortestosterone. These products are sold as over-the-counter products under the 1994 Dietary Supplement Health and Education Act, although the recently “Anabolic Steroid Control Act of 2004” was intended to ban these substances. Global distribution via the internet has resulted in a huge international commercial success for products including AD, 19-norandrostenedione and dehydroepiandrosterone. The use of these substances constitutes a doping offence according to WADA regulations [4].

The target analyte in this study, androst-4-ene-3,6,17-trione, is an anabolic steroid exhibiting aromatase-inhibiting properties in several in-vitro experiments [5-7]. This steroid is new on the nutritional supplement markt and is advertised as an anti-estrogenic agent to be used by athletes to treat gynecomastia. A recent qualitative GC-MS-method has revealed that androst-4-ene-3,6,17-trione is metabolised to androst-4-ene-6α-ol-3,17-dione and androst-4-ene- 6α, 17β-diol-3-one [8]. Using this GC-MS method an additional derivatisation step was needed to determine the position of the hydroxyl function at the C-6. Until now, quantitative data is lacking and no method has been described for the direct detection of 6-hydroxy-steroids that retains the stereochemical information at the C-6 atom, although these compounds have been reported previously as metabolites of several steroids [9-12].

Experimental

Chemicals and reagents

6-oxo-AD, 6α-OH-AD, 6β-OH-AD, 6α-OH-T and 6β-OH-T were purchased from Steraloids (Newport, RI, USA). The internal standard (desoximetasone) and the β-glucuronidase preparation (type HP-2; ≥ 7500 Units/ml sulphatase, ≥ 92500 Units/ml glucuronidase) were purchased from Sigma (Bornem, Belgium). Analytical grade sodium acetate, potassium carbonate, sodium hydrogen carbonate, glacial acetic acid and diethylether were from Merck (Darmstadt, Germany), HPLC grade acetonitrile was from Biosolve (Geel, Belgium) and HPLC grade water was from Fischer (Loughborough, UK). The nutritional supplement 6-OXO® was bought from Ergopharm (Champaign, USA) via internet. The content of one capsule was 100 mg 6-oxo-AD. The manufacturer's recommended daily dosage was 3- 6 capsules in cycles of 4-6 weeks.

Excretion study

The study was performed with four healthy male volunteers. The study protocol was reviewed and approved by the ethical committee of Ghent University Hospital

(UZGent, Project EC/2005-81/sdp). Each volunteer signed a statement of informed consent. One capsule was taken in the morning. Samples were collected quantitatively before (0h) and 2, 4, 6, 8, 10 and 12h after administration. Additional samples were taken after 24, 30, 36 and 48h.
All urine samples were either analysed directly or stored at -20°C awaiting analysis. Urinary pH, volume and density were measured and samples were analyzed in duplicate. When necessary, urine samples were diluted with blank urine in order to obtain concentrations in the range of the calibration curve.

Sample treatment

Sample clean-up was kept to a minimum and a previously described method for the extraction of anabolic steroids and analysis by GC-MS was used [8].
After the addition of the internal standard solution (50 µl desoximetasone, 10 µg/ml) to 3 ml of urine, 1 ml sodium acetate buffer (pH 5.2) and 50 µl β-glucuronidase preparation were added and the samples were hydrolysed for 2.5h at 56°C. After cooling, 100 mg of a solid buffer containing sodium hydrogen carbonate and potassium carbonate (2:1 w/w) was added to the hydrolysate. Liquid-liquid extraction was performed by rolling for 20 min with 5 ml diethylether. After centrifugation (1200*g*) the organic layer was separated and evaporated under OFN. The residue was dissolved in 200 µl of the initial mixture of the mobile phase.
For the determination of the percentage of unconjugated metabolites, the samples were analysed similarly, except for the hydrolysis step.

Method validation

A six-point calibration curve was established between 5 ng/ml and 1000 ng/ml (5, 10, 50, 100, 500, 1000 ng/ml) for 6-oxo-AD, 6α-OH-AD and 6α-OH-T in blank urine. Each concentration was analyzed in triplicate, the averages were used to construct the calibration curve. The area ratios of the protonated molecules of the compounds of interest and the protonated molecular ion m/z 377 of the internal standard were plotted versus the concentration.

The precision and inaccuracy of the method were tested at three levels (5, 100 and 1000 ng/ml). Precision was assessed as the percentage RSD of both repeatability (within-day) and reproducibility (between-day and different analysts) for a selected compound and level. Maximum allowed tolerances for reproducibility and repeatability can be calculated from the Horowitz-equation $RSD_{max} = 2^{(1-0.5\log C)}$ (C = concentration (µg/ml)*10^{-6}). The maximum allowed tolerances for repeatability and reproducibility are $2/3RSD_{max}$ and RSD_{max}, respectively [13].

Inaccuracy (expressed as mean error) was defined as the difference between the calculated amount and the specified amount for the selected compound and expressed as a percentage

The LOQ of the method was defined as the lowest concentration where acceptable reproducibility and inaccuracy could be guaranteed. Selectivity was tested by analysing several structurally related and other routinely screened doping agents, including corticosteroids and anabolic steroids. Concentrations of these mixtures were 1 µg/ml.

Specificity was tested during the validation procedure where ten blank urines were extracted and analysed as described above.

Blank urine, a system blank and a quality control sample (spiked at 100 ng/ml) were analyzed concurrently in each batch of samples.

Extraction recovery was calculated for all compounds by extracting urine samples (*n*= 6), spiked at 3 levels (5, 100, 1000 ng/ml), together with negative urine samples (*n*=6). Extracts of the negative urine samples were then spiked at the same levels simulating a 100% recovery. Both sets of samples were then evaporated and analysed with the described LC-MS method. The obtained peak areas of the two sets of samples were compared.

Instrumentation

Separation of the compounds was performed on a Nucleosil C18 column, 100 mm x 3 mm, 5 µm equipped with a guard column 10 mm x 2 mm (both from Varian, Sint-Katelijne-Waver, Belgium) using a P4000 pump and a model AS3000 autosampler (TSP, San Jose, USA) The mobile phase consisted of acetonitrile and 1 % acetic acid in water. Gradient elution at a flow rate of 0.3 ml/min was as follows: 70% acetic acid

(1%) for 2 min, followed by a linear decrease to 30% in 3 min, isocratic for 5 min, followed by an increase in 0. 5 min to 70% acetic acid (1%) which was maintained for 8 min before the next injection (equilibration time). The total run time of the method was 18 min. The injection volume was 50 µl.

Ionisation of the analytes was carried out on a LCQ-Deca instrument (Thermo, San Jose, USA) using APCI in the positive ionisation mode. The corona discharge current was set at 5 µA. The capillary temperature and evaporator temperature were maintained at 150 ºC and 300 ºC, respectively. The drying gas (nitrogen, LASAL2001, Air Liquide, Destelbergen, Belgium) was kept at 80 units while the auxiliary gas (nitrogen) was set at 10 units. The capillary voltage was maintained at 10 V.

For the MS^2 experiments the collision energy was set at 35 %. The isolation width was set at 3.0 and an activation q of 0.250 was applied.

Results and Discussion

Mass spectrometry

Flow injection analysis was performed to determine diagnostic ions. For each tested compound a solution of 5 µg/ml was infused at a flow rate of 10 µl/min. APCI is less susceptible to matrix effects [14, 15] and was preferred as interface. Positively charged $[M+H]^+$ ions were observed for all compounds in full scan MS.

Additionally all compounds were tested in full scan MS^2 and similar fragmentation patterns were observed (Figure 1). For all compounds consecutive loss of water $[M+H-H_2O]^+$ and $[M+H-2H_2O]^+$, a very common fragmentation pattern, was observed. The intensive fragmentation in MS^2 substantially reduces the signal to noise ratio for the characteristic ions compared to the protonated molecular ion in full scan MS. Moreover the most abundant product ions $[M+H-H_2O]^+$ and $[M+H-2H_2O]^+$ lack specificity in the hydrolysed samples, a phenomenon previously noticed for beclomethasone [16]. Better sensitivity was observed in full scan MS. Consequently quantification was done in full scan APCI+-mode and MS^2 was only used for qualitative purposes and confirmation.

Extraction and chromatography

For chromatography a reversed phase column was preferred, since these columns show good selectivity for the separation of steroids [17,18]. 6α-OH-T (RT 4.75 min), 6α-OH-AD (RT 6.32 min) and 6-oxo-AD (RT 7.83 min) exhibited well separated peaks under the chromatographic conditions described.

Usually, hydrolysis of urine samples can lead to an increase in matrix background. As a consequence an isocratic step of two minutes was included allowing non-retained and/or poorly retained matrix compounds resulting from the hydrolysis to elute in the early stage of the chromatographic run.

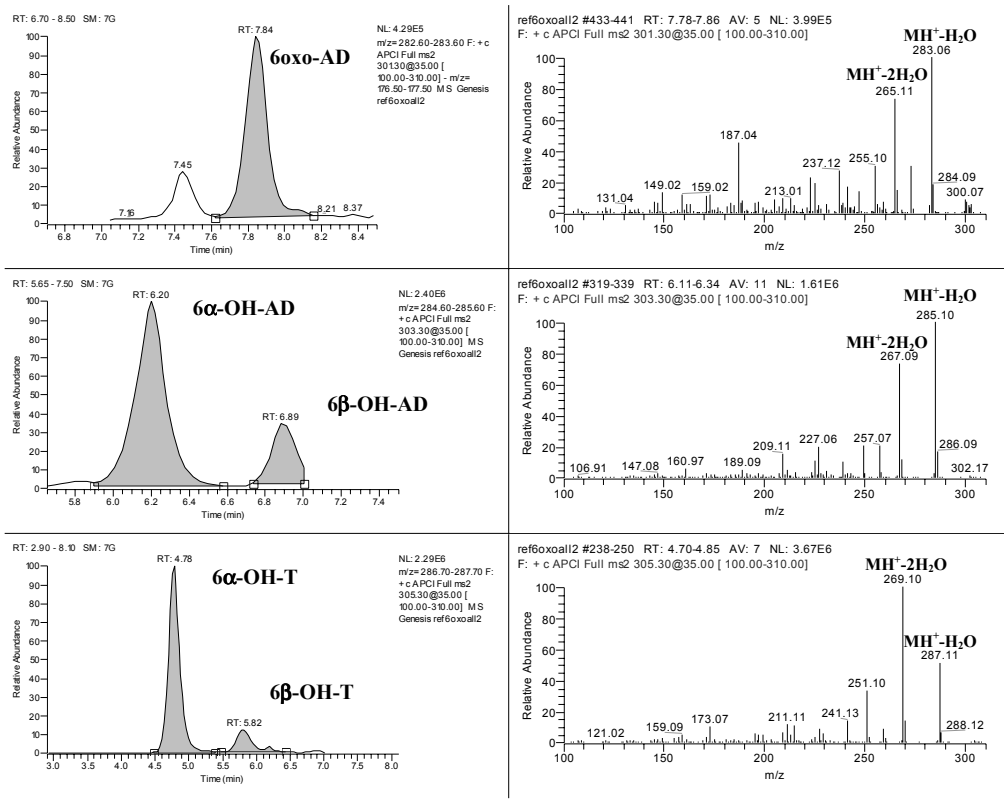

Figure 1: Extracted ion chromatograms and mass spectra of 6oxo-AD (m/z 283), 6α-OH-AD (m/z 285) and 6α-OH-T (m/z 287) in a reference mixture (100 ng/ml).

Table 1:
Inaccuracy (between-day), repeatability, reproducibility and tolerance limits of the LC-MS method for 6-oxo-androstenedione (6-oxo-AD), 6α-OH-androstenedione (6α-OH-AD), 6α-OH-testosterone (6α-OH-T).

Conc. [ng/ml]	Inaccuracy [%] *n=18*			Repeatability [%] *n=6*			Reproducibility [%] *n=18*			RSD_{max} [%]	$2/3\ RSD_{max}$ [%]
	6-oxo-AD	6α-OH-AD	6α–OH-T	6-oxo-AD	6α-OH-AD	6α–OH-T	6-oxo-AD	6α-OH-AD	6α–OH-T		
5	-14.8	9.4	-4.1	9.0	14.5	14.9	11.8	14.9	13.3	35	23
100	11.3	-5.7	2.4	3.5	6.1	4.1	7.4	8.2	7.1	22	15
1000	-1.3	-1.6	-3.2	6.1	4.1	5.2	8.4	9.5	7,4	16	10

Method validation

Using a least square fit, good linearity ($r^2 \geq 0.998$) was observed for all compounds in the range 5 - 1000 ng/ml. None of the calibration curves was forced through the origin and for the regression calculation a weighing factor of 1/x was used for all data points.
The results for precision and inaccuracy are summarised in Table 1.
As shown in Table 1, the tolerances were never exceeded for either repeatability or reproducibility. Deviation of the mean measured concentration from the theoretical concentration (inaccuracy) for all compounds were below the acceptable threshold of 15% [19] for all the levels.
Regarding the selectivity, interference from other monitored doping agents could not be found. In addition analysis of 10 different blank control urine samples did not result in the detection of background noise, proving the specificity of the method.
The LOQ of the method was 5 ng/ml. The LOD, arbitrarily set at ½ of the LOQ, was 2.5 ng/ml. Extraction recoveries for the different compounds are given in Table 2. Moderate to good recoveries were obtained for all compounds.

Table 2: Extraction recoveries for 6-oxo-AD, 6α–OH-AD and 6α-OH-T.

Conc. [ng/ml]	Recovery (%) $n = 6$		
	6-oxo-AD	6α-OH-AD	6α-OH-T
5	92.4 ± 16.3	83.6 ± 18.6	82.8 ± 9.6
100	97.3 ± 0.9	84.8 ± 4.8	66.0 ± 5.2
1000	78.0 ± 2.0	90.0 ± 6.4	69.9 ± 3.8

Values are presented as mean ± standard deviation (n=6).

Excretion studies

Qualitative results

Separation of isomers is unpredictable in both GC-MS and LC-MS and must be tested experimentally. Moreover comparison between both techniques is difficult because well separated compounds on GC-MS can co-elute on LC-MS and vice versa. Derivatisation with a mixture of MSTFA/NH_4I/ethanethiol is most frequently used in GC-MS doping analysis as this mixture results in the in-situ formation of trimethylsilyliodide, the strongest trimethylsilylating agent, and in the formation of enol-TMS-ether-derivatives [20]. Unfortunately, for 3-keto-4-ene-steroids, trimethylsilylation with this mixture results in 3,5-dienol formation and loss of stereochemical integrity at C-6 [21]. Hence, a different derivatisation procedure was needed for the GC-MS determination of the stereochemical configuration of the 6-hydroxy metabolites of 6-oxo-AD [8].

By using the LC-MS method described here, the isomers of 6ζ-OH-AD and 6ζ-OH-T were well separated. Hence the 6-hydroxy isomers of androstenedione and testosterone, previously detected as metabolites of 6-oxo-AD [8], could be readily identified as 6α-isomers. Taking into account the metabolism of androst-4-ene-3,17-dione to testosterone in humans [11, 22, 23] and the in-vivo 6α-hydroxylation of androst-4-ene-3,17-dione [11,12], the metabolic pathway shown in Figure 2 is suggested for 6-oxo-AD.

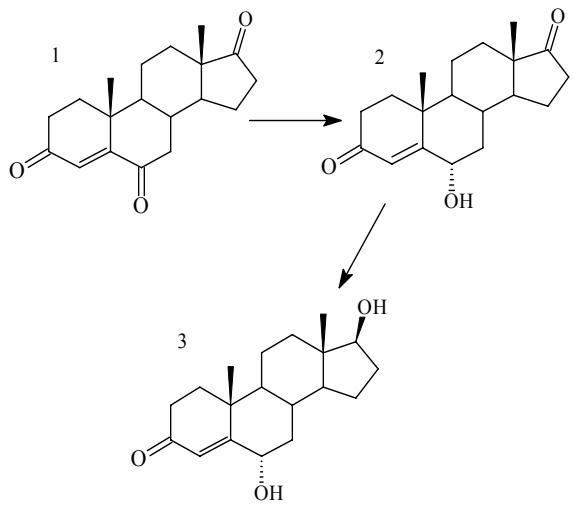

Figure 2. Suggested in-vivo metabolic pathway from androst-4-ene-3,6,17-trione (1) to androst-4-ene- 6α, 17β-diol-3-one (3) via androst-4-ene-6α-ol-3,17-dione (2).

Using the described method, 6β-hydroxy isomers were not detected similar as for androst-4-ene-3,17-dione where the 6β-hydroxy isomers were only detected as in-vitro metabolites [12].

Quantitative results

The parent compound 6-oxo-AD could be detected in all urine samples. The urinary excretion profiles are shown in Figure 3a.
Maximum urinary concentrations of 6-oxo-AD were obtained 2-4 h after administration. The concentrations ranged from 470 ng/ml to 1900 ng/ml and 6-oxo-AD was not longer detectable 30 h post administration.
6-oxo-AD was mainly excreted conjugated. Only 12.6 ± 2.8 % (n=6) of 6-oxo-AD was excreted unconjugated. Cumulative excretion data (total fraction) are shown in Figure 3b indicating a rapid clearance after oral intake of a capsule of 6-oxo-AD.

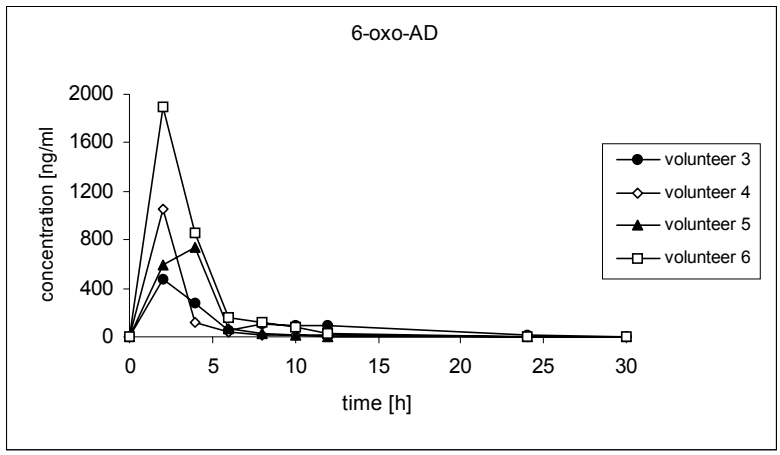

Figure 3(a)

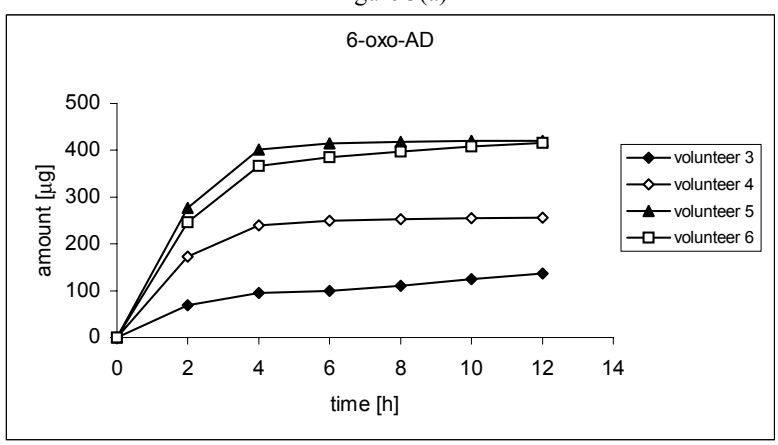

Figure 3(b)

Figure 3: Urinary concentrations (a) and cumulative excretion (b) of 6-oxo-AD after administration of one capsule of 6-OXO®.

The major metabolite of 6-oxo-AD was identified as 6α-OH-AD and maximum urinary concentrations were detected 2-4 hours after intake (Figure 4a). The concentrations ranged from 32 µg/ml up to 55 µg/ml. 6α-OH-AD could only be detected in the conjugated fraction up to 30 h post administration and it was also rapidly cleared from the body.

Cumulative data of 6α-OH-AD are presented in Figure 4b.

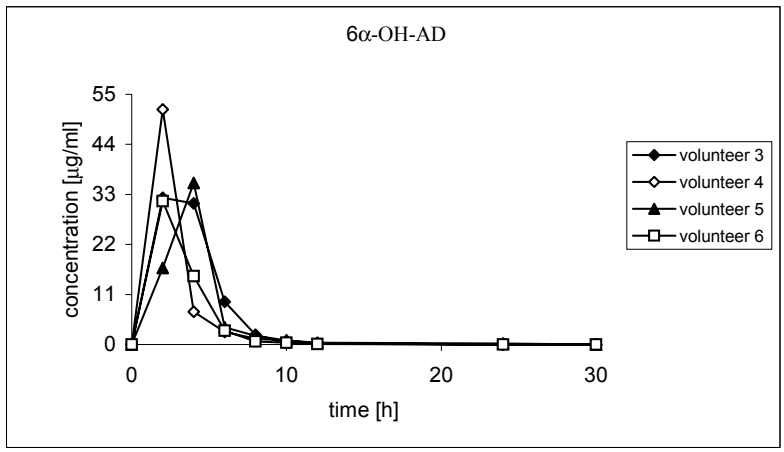

Figure 4(a)

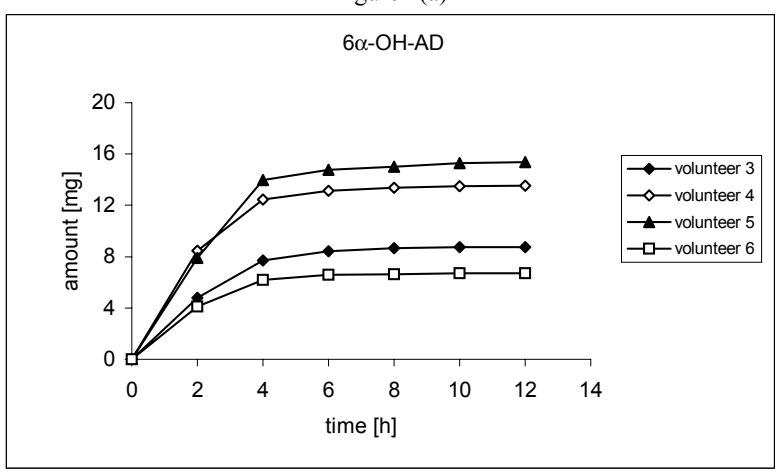

Figure 4(b)

Figure 4: Urinary concentrations (a) and cumulative excretion (b) of 6α-OH-AD after administration of one capsule of 6-OXO®.

6α-OH-T was identified as a minor metabolite and remained detectable up to 24 hours after intake (Figure 5a). Maximum urinary concentrations were reached 2-4 h after intake. Concentrations varied between 250 and 500 ng/ml. Cumulative excretion data of 6α-OH-T are presented in Figure 5b. Similar to 6α-OH-AD, 6α-OH-T was only detected in the conjugated fraction (results not shown). Preliminary experiments with

glucuronidase from E. Coli instead of H. Pomatia indicate that 6-oxo-AD and metabolites are excreted as glucuronide conjugates.

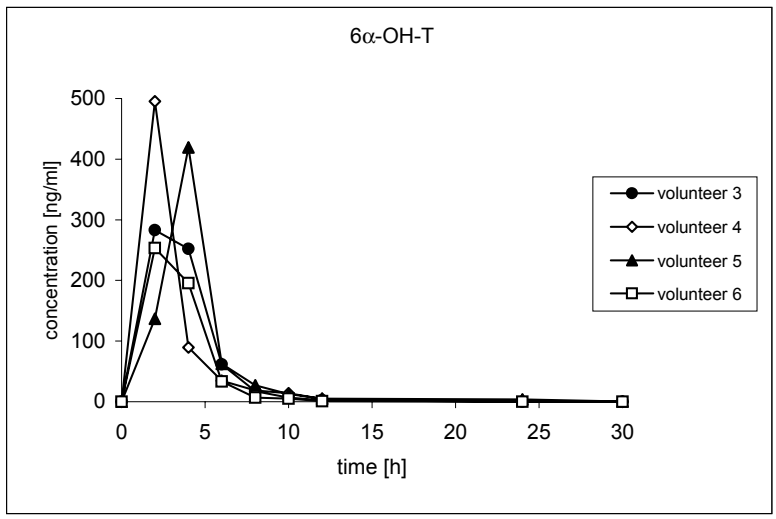

Figure 5(a)

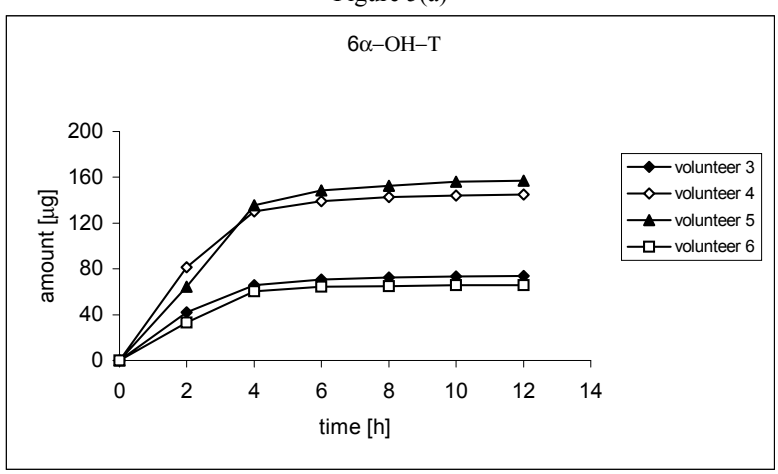

Figure 5(b)

Figure 5: Urinary concentrations (a) and cumulative excretion (b) of 6α–OH–T after administration of one capsule of 6-OXO®.

Conclusions

A sensitive LC-APCI/MS method for the quantification of 6-oxo-AD, 6α-OH-AD and 6α-OH-T in urine was developed and validated. The method enabled the differentiation between 6α- and 6β- hydroxy isomers of 3-keto-4-ene-steroids.
Urine samples after the administration of 6-oxo-AD were analysed. Low concentrations of the parent drug 6-oxo-AD were detected in the samples up to 30 h post administration. 6α-OH-AD was identified as the major metabolite of 6-oxo-AD and 6α-OH-T was found to be a minor metabolite. 6-oxo-AD was predominantly detected in the conjugated fraction while 6α-OH-AD and 6α-OH-T were only detected in the conjugated fraction.
Using the described method, misuse of 6-oxo-AD can be detected until 30 h post administration of a single dose of 100 mg.

Acknowledgements

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Chapter V: Stimulants

1. Introduction

Therapeutic use

Stimulants cover a broad category of substances, including those prescribed for medical conditions, those manufactured for illicit substance abuse and those found in over-the-counter herbal extracts, beverages, and cigarettes. Commonly known stimulants are amphetamine, ephedrine, nicotine, caffeine, cocaine, XTC and strychnine.

Chinese physicians have been using *ephedra* (a stimulating herb) for more than 5,000 years to treat common colds, coughs, asthma, headaches, and hay fever. In general, many stimulants are synthetic analogues of naturally occurring compounds [1].

Stimulants have been used intensively for the treatment of depression and as appetite suppresant. However the clinical use of stimulants has some severe side effects including addiction and paranoia. They are also frequently used as 'recreational' drugs (XTC, speed, cocaine). During the 1980s Captagon® (fenethylline) was reported as a popular drug among students.

Nowadays, improvements in the area of stimulant pharmacology resulted in a class of compounds which increase alertness without the addictive potential of traditional stimulants. They also have minimal effect on sleep structure and do not result in rebound hypersomnolence or "come down" effects. Currently, there are two stimulants in this class: modafinil and adrafinil, marketed as Provigil® and Olmifon®, respectively.

Other stimulants still frequently used are pseudoephedrine (Sinutab®), used for the treatment of nasal congestion and in coughing syrups (Actifed®) and methylphenidate (Rilatine®), in the treatment of ADHD [2]. The structures of some selected stimulants are presented in Figure 1.

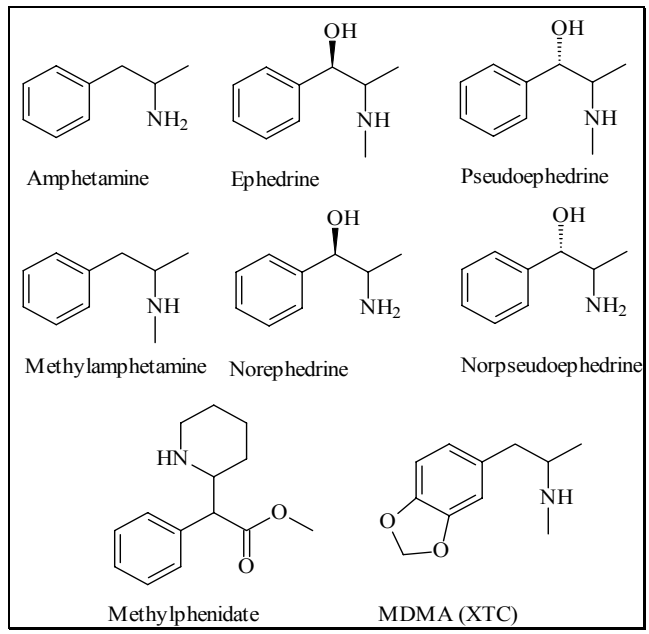

Figure 1: Structure of selected stimulants.

Use as doping agents

In sports requiring intense anaerobic exercise, amphetamines prolong the tolerance to anaerobic metabolism [3]. These agents reduce fatigue during endurance events and training sessions. Stimulants are also used to improve concentration and to increase aggression. Athletes such as wrestlers and jockeys have also used these agents as appetite suppressants to control weight. Consequently they are on the list of prohibited substances published by WADA [4].

Caffeine, a mild stimulant, is the most widely used psychoactive drug in the world and is also used to reduce fatigue and prolong endurance. It is present in soft drinks, coffee, tea, chocolate and numerous prescription and over-the-counter drugs. From January 1st 2004 caffeine has been removed from the WADA list of prohibited substances. A comparative study between results obtained in 2004 and before the removal of caffeine from the WADA (World Anti-Doping Agency) doping list indicate that average

caffeine concentrations decreased after the withdrawal of caffeine from the list of prohibited substances [5].

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2. Screening for amphetamine and amphetamine type drugs

Adapted from:

Deventer K, Van Eenoo P, Delbeke FT.
Screening for amphetamine and amphetamine type drugs in doping analysis by liquid chromatography-mass spectrometry.
Rapid Commun. Mass Spectrom. 2006; **20**: 877.

Abstract

A selective and sensitive method for the qualitative screening of urine samples for 27 amphetamine and amphetamine-type drugs in the field of doping analysis is described.
The method consists of a liquid-liquid extraction with diethylether at pH 14 and analysis of the extracts with a LCQ-Deca® instrument equipped with APCI operated in positive ionisation mode. The total run time was 15 minutes. All compounds were analysed in MS^2 or MS^3. The detection limit for all compounds was lower than 25 ng/ml except for chlorphentermine (LOD: 250 ng/ml)

Introduction

Amphetamine was first introduced as a synthetic analogue for ephedrine in the treatment of asthmatic and allergic affections and was first synthesized by Edeleano [1]. Amphetamine has a marked effect on mental function and behaviour, producing excitement and euphoria, reducing sensation of fatigue and increasing motor activity caused by the release of noradrenaline [2]. Hence, soon after its introduction it gained popularity as a general stimulant. After the discovery of its ability to suppress appetite, amphetamine became a popular dietary supplement as well. This molecule has been a target for molecular modification in order to accentuate or abolish some of its effects or to hamper their detection.
Amphetamines are addictive and can result in paranoid psychosis when increasing doses are administered [3].
In sports requiring intense anaerobic exercise, amphetamines prolong the tolerance to anaerobic metabolism [4]. Consequently they are on the list of prohibited substances published by WADA [5]. For some ephedrine type compounds a threshold in urine is applied [5].
Because of their volatility amphetamines can be analysed underivatized using GC. In doping analysis GC-NPD is the preferred technique for screening purposes [6-8], although selectivity and sensitvity can be improved by applying GC-MS [7,9]. Unfortunately a derivatization step is required when GC-MS is used because the mass spectra of underivatized stimulants exhibit base peaks at low masses and minor ion

intensities at higher masses [9,10]. In doping analysis TFAA or MBTFA in combination with MSTFA is commonly used for this purpose [7].
LC-MS does not require a derivatization step. Moreover in the last decade LC-MS has proven to be the ideal technique for the detection of polar compounds [11]. Consequently several methods using LC-MS for the detection of amphetamines in different tissues were recently reported [12-16].
Until now, no comprehensive LC-MS screening method for the detection of these substances, suitable in doping analysis, has been reported yet.
The aim of this study was to develop such a screening method for the detection of amphetamine and amphetamine-type drugs as an alternative for the existing GC-methods.

Experimental

Chemicals and reagents

Standards

3-bromophenethylamine, dimethylamphetamine HCl, mephentermine sulphate and phendimetrazine HCl were purchased from Sigma-Aldrich (Bornem, Belgium). MDA, MDEA and MDMA were a kind gift from the Portuguese doping control laboratory. Fencamfamine HCl, norephedrine HCl, norpseudoephedrine HCl, pseudoephedrine HCl and methamphetamine HCl were purchased from Merck (Darmstadt, Germany), pipradrol HCl from Merrell-Dow (Cincinnati, Ohio, USA) and amphetamine sulphate from GlaxoSmithKline (Philadelphia, USA). Phenmetrazine and prolintane HCl were a gift from Boehringer & Sohn (Ingelheim am Rhein, Germany). Heptaminol HCl was purchased from Ets. A De Bournonville (Braine L'Alleud, Belgium), norfenfluramine HCl from Eutherapie Benelux (Brussels), ephedrine HCl from Hoechst AG (Frankfurt, Germany) and fenfluramine HCl from Laboratoires Servier (Orleans, France). Methylephedrine HCl was a gift from from Laboratoire G.A. (Cochard, France), phentermine HCl from NV Certa Noville (Mehaigne, Belgium), nikethamide from Ciba-Geigy (Groot-Bijgaarden, Belgium) and mefenorex from Produits Roche (Brussels). Chlorphentermine HCl was purchased from Tropon GmbH (Köln,

Germany). Isopropylhexedrine was purchased from Veride (Diegem, Belgium) and ethylamphetamine HCl from Will-Pharma Benelux (Brussels).

Reagents

Potassium hydroxide p.a. was purchased from Merck (Darmstadt, Germany). Methanol HPLC-grade was purchased from Acros-Organics (Geel, Belgium), diethylether p.a from Biosolve (Valkenswaard, The Netherlands) and formic acid and HPLC-grade water from Fischer Scientific (Hampton, United Kingdom).
Gases used in mass spectrometry were helium (Alphagaz-grade) and nitrogen (LASAL2001-grade) both from Air Liquide (Desteldonk, Belgium).
Methanolic HCl (1M) was prepared by the addition of 3.9 g acetyl chloride (Sigma, Bornem) using a dropping funnel during a period of 20 minutes with stirring, into 50 ml of methanol p.a. (Acros Organics, Geel, Belgium) cooled to 0°C. The solution is stored between 0 and 8 °C.

Sample treatment

The internal standard (IS) solution (50 µl 3-bromophenethylamine, 10 µg/ml) was added to 2 ml urine, followed by addition of 0.5 ml of 5M aqueous potassium hydroxide. Liquid-liquid extraction was performed by rolling for 10 min with 5 ml diethylether. After centrifugation (1200*g*) the organic layer was transferred into a new tube and 100 µl of methanolic HCl (1M) was added. Finally the organic layer was evaporated until dry under oxygen free nitrogen (OFN) at 40 ºC. The remaining residue was dissolved in 200 µl of the initial mobile phase. 50 µl was injected into the HPLC-system using push loop filling.

Method validation

The validation was carried out following Eurachem guidelines [17].
Ten urine samples, declared negative after routine doping analysis, were spiked at 8 different levels. Final concentrations were 500, 250, 100, 50, 25, 10, 5 and 1 ng/ml. The

samples were extracted as described above.
The LOD was defined as the lowest level at which a compound could be identified in all 10 urines with diagnostic ions present with a signal to noise ratio greater than 3. Selectivity was tested by analysing several doping agents which are routinely screened for including narcotics, corticosteroids, anabolic steroids and diuretics. Concentrations of these mixtures were 1 µg/ml.
Specificity was tested during the validation procedure. Therefore, ten blank urines were extracted and analysed as described above.

Recovery

Recovery was tested at pH 9.2 and 14. These pH values are commonly used in doping analysis for the extraction of basic compounds [7].
For this purpose urine samples (n = 6) were spiked with the different compounds at 25 ng/ml except for chlorphentermine (250 ng/ml) and extracted together with non spiked urine samples (n = 6). The extracts of the non spiked samples were then spiked simulating a 100% recovery. After adding methanolic HCl both sets of samples were evaporated and analysed to evaluate recovery. Peak areas in the two sets were compared.

Chromatography

The HPLC system consisted of a P4000 quaternary pump and an AS 3000 autosampler with a 100 µl sample loop (all from Thermo Separation Products, Thermo, San Jose, CA, USA).
An Omnispher C18 column 50 x 3 mm, 3 µm (Chrompack, Antwerp, Belgium), protected with a guard column 10 x 2 mm (Chromsep, Antwerp) was used for chromatographic separations. The column was maintained at a temperature of 35ºC. The mobile phase consisted of 0.1% formic acid (solution A) and MeOH. Gradient elution at a flow rate of 0.4 ml/min was as follows: 90% A for 2 minutes, linear to 26% in 8 min, followed by an increase to the initial concentration of 90% A in 0.5 min. Equilibration time was 4.5 min, total run time 15 minutes.

Mass spectrometry

The LC effluent was pumped to an LCQ-Deca® ion trap mass spectrometer instrument (Thermo, San Jose, CA, USA) equipped with an APCI source operated in the positive ionisation mode.

The APCI corona discharge current was set to 5 µA. The capillary temperature and the vaporizer temperature were 120 and 350 ºC, respectively. The sheath gas flow rate was set at 50 arbitrary units. No auxilliary gas was used.

Flow injection analysis was performed to determine diagnostic ions. For each tested compound a solution of 5 µg/ml was infused at a flow rate of 10 µl/min.

In MS^2 experiments the isolation width was set at 3.0, the activation q at 0.250 and the activation time at 30 ms. The number of microscans was set to 1 and the injection time to 200 ms. The collision energy was set allowing 100 % fragmentation of the precursor ion.

Results and Discussion

Method development

Despite extensive hepatic metabolism, a significant percentage of the amphetamines is excreted unaltered [18, 19]. Hence parent compounds were used as target compounds in the developed screening method. LC-MS allows for the direct analysis of aqueous samples (urine, plasma) reducing sample preparation, cost and time [12]. However, direct analysis of non- extracted urine samples inevitably results in reduced sensitivity. Therefore LLE was preferred as clean up step [7, 20].

Moderate to good recoveries were achieved at pH 9.2 for most compounds. Generally, best recoveries were obtained at pH 14. (Table 1). Moreover heptaminol could only be extracted at pH 14 albeit that, even at pH 14, heptaminol exhibited the lowest recovery

Table 1: Extraction recoveries at pH 9.2 and 14.

	Recovery (%) *n = 6*	
Compound	pH 9.2	pH 14
3-Bromophenethylamine*	-	-
Amphetamine	75.2 ± 4.6	99.3 ± 7.4
Chlorphentermine	98.1 ± 15.5	97.2 ± 2.6
Dimethylamphetamine	104.0 ± 7.1	94.5 ± 5.4
Ephedrine	56.8 ± 4.1	91.7 ± 7.0
Ethylamphetamine	88.2 ± 6.8	95.8 ± 4.3
Fencamfamine	88.0 ± 5.2	103.3 ± 4.6
Fenfluramine	122.5 ± 35.6	100.3 ± 6.4
Heptaminol	-	36.4 ± 7.1
Isopropylhexedrine	86.0 ± 5.0	100.1 ± 3.7
MDA	80.9 ± 6.0	87.4 ± 13.6
MDEA	94.5 ± 10.2	97.5 ± 11.0
MDMA	80.0 ± 6.3	96.6 ± 11.0
Mefenorex	95.5 ± 3.0	101.4 ± 6.2
Mephentermine	79.3 ± 5.1	104.4 ± 5.9
Methamphetamine	84.3 ± 10.2	94.3 ± 7.0
Methoxyphenamine	90.3 ± 1.8	102.5 ± 4.4
Methylephedrine	97.7 ± 9.1	91.6 ± 7.0
Nikethamide	64.1 ± 6.6	72.8 ± 25.7
Norephedrine	43.9 ± 11.4	76.4 ± 8.1
Norfenfluramine	102.1 ± 18.8	103.6 ± 2.5
Norpseudoephedrine	43.9 ± 11.4	55.0 ± 11.9
Phendimetrazine	89.9 ± 3.8	98.4 ± 4.8
Phenmetrazine	91.0 ± 5.5	88.4 ± 5.3
Phentermine	96.0 ± 3.9	95.9 ± 10.1
Pipradrol	97.5 ± 4.5	96.3 ± 5.1
Prolintane	83.2 ± 7.4	87.1 ± 9.3
Pseudoephedrine	56.8 ± 4.1	77.5 ± 1.9

* Internal Standard

Values are presented as mean ± standard deviation.

of all compounds. Nevertheless the obtained LOD at pH 14 is compliant with the MRPL set by WADA [21].
In doping analysis diphenylamine is commonly used as internal standard for the GC-analysis of amphetamine type drugs [7, 20]. Since its chemical structure substantially differs from amphetamine, 3-bromophenethylamine was used instead.
The volatility of amphetamines is commonly known. To avoid undesired loss of compounds, methanolic HCl was added to the organic phase before the evaporation step [22] although reproducible results have been reported without the use of HCl [23].

Both ESI and APCI-interfaces were tested for all compounds. Amphetamines contain an amine function which can be easily protonated. Hence very abundant protonated molecular ions $[M+H]^+$ were observed for all compounds with both interfaces. Deprotonated molecular ions were not detected in negative ionisation mode. Ultimately, APCI was prefered as interface based on its robustness towards matrix interferences [24].
Gases and temperature of the heated capillary and vaporizer were carefully tuned. When the sheath gas flow was altered above 50 units, gradual suppression of the signal was observed. The use of auxillary gas resulted in the loss of signal intensity as well and its use was avoided. Whereas the response of the signal was not significantly affected by the vaporizer temperature, the temperature of the heated capillary had to be limited to 120 ºC. Above this temperature undesired fragmentation was observed.
Because tandem mass spectrometry often results in improved sensitivity this technique was applied for most compounds. Product ions resulting from MS^2 and MS^3 experiments are presented in Table 2.

Table 2: MS^2 data from infusion experiments.

MS^2 Compound	PI $[M+H]^+$	CE	Product Ions (Relative abundance)
3-Bromophenethylamine	200	25	183(100)
Amphetamine	136	25	119(100)
β-Phenethylamine	122	25	105(100)
Chlorphentermine	184	25	167(100)
Dimethylamphetamine	164	30	119(100), 91(15)
Ephedrine	166	25	148 (100)
Ethylamphetamine	164	30	119(100), 91(12)
Fencamfamine	216	25	171(100), 159(10), 143(12), 129(32)
Fenfluramine	232	30	187(100), 159(28)
Heptaminol	146	25	128 (100)
Isopropylhexedrine	156	30	125 (100), 83(70)
MDA	180	30	163(100)
MDEA	208	30	163(100)
MDMA	194	25	163(100)
Mefenorex	212	30	119(100), 91(40)
Mephentermine	164	30	133(100)
Methamphetamine	150	30	119(100), 91(8)
Methoxyphenamine	180	30	149(100)
Methylephedrine	180	30	162(100)
Nikethamide	179	35	108(100), 72(18)
Norephedrine	152	25	134(100)
Norfenfluramine	204	25	187(100)
Norpseudoephedrine	152	25	134(100)
Phendimetrazine	192	35	174(25), 159(6),148(100), 147(90), 119(18), 100(14), 91(23), 86(6), 74(14)
Phenmetrazine	178	35	160(100),143(25), 134(70), 119(40), 117(20), 91(34)
Phentermine	150	30	133(100)
Pipradrol	268	25	250(100)
Prolintane	218	35	105(80), 91(100)
Pseudoephedrine	166	25	148(100)

PI: Precursor Ion, CE: Collision Energy

Table 2 (continued): MS^3 data from infusion experiments.

MS^3			
Compound	PI	CE	Product Ions
Norephedrine	134	25	117(100)
Norpseudoephedrine	134	25	117(100)

PI: Precursor Ion, CE: Collision Energy

Few product ions were observed during infusing experiments. For most substances the most abundant signal in MS^2 was generated by the loss of the amine moiety. More fragmentation was found for fencamfamine, phenmetrazine and phendimetrazine (Table 2).

For compounds derived from ephedrine the sole detected ion was generated by the loss of H_2O. In MS^2, criteria for selectivity were not fullfilled for norephedrine and norpseudoephedrine. In MS^3 however selectivity was fulfilled for these two compounds. Due to the short chromatographic run, coelution was observed for different compounds (Figure 1) and up to 7 transitions had to be monitored simultaneously resulting in few datapoints per substance. To enhance the number of datapoints the default instrument setting of 2 microscans per data point was reduced to 1 microscan. In this way the instrument generated one datapoint for every microscan. However, such mass spectrometric data is not averaged and the technique can only be used for screening purposes.

The influence of the organic modifiers MeOH and ACN was also tested. Although no difference was observed between the two solvents, the use of MeOH was prefered as it allowed the isomers ephedrine-pseudoephedrine and norpseudoephedrine-norephedrine to be partially separated (Figure 1). With acetonitrile such a separation was not observed. Partial chromatographic resolution can be used for screening purposes. In the confirmation procedure however, separation of these 4 isomers is mandatory [19, 25, 26].

Method Validation

Detection limits are given in Table 3. LODs were below 25 ng/ml except for chlorphentermine (250 ng/ml) and were in compliance with the MRPL of 500 ng/ml for stimulants imposed by WADA [21].

Ion chromatograms obtained after analysis of a quality control urine spiked with all compounds at a concentration below WADA's MRPL are given in Figure 1.

The described method is very selective as no interferences were detected when other doping products including narcotics, corticosteroids and anabolic steroids were analysed.

Specificity was satisfactory as no interfering substances at the appropriate retention times were found when 10 blank urines were analysed.

Table 3: Urinary detection limits at pH 14

Compound	LOD (ng/ml)	Compound	LOD (ng/ml)
3-Bromophenethylamine*	/	Mephentermine	25
Amphetamine	10	Methamphetamine	25
β–Phenethylamine**	/	Methoxyphenamine	5
Chlorphentermine	250	Methylephedrine	25
Dimethylamphetamine	10	Nikethamide	25
Ephedrine	25	Norephedrine	25
Ethylamphetamine	10	Norfenfluramine	10
Fencamfamine	10	Norpseudoephedrine	10
Fenfluramine	1	Phendimetrazine	25
Heptaminol	25	Phenmetrazine	25
Isopropylhexedrine	10	Pipradrol	10
MDA	25	Phentermine	25
MDEA	1	Prolintane	1
MDMA	1	Pseudoephedrine	25
Mefenorex	10		

* Internal Standard

**Endogenous

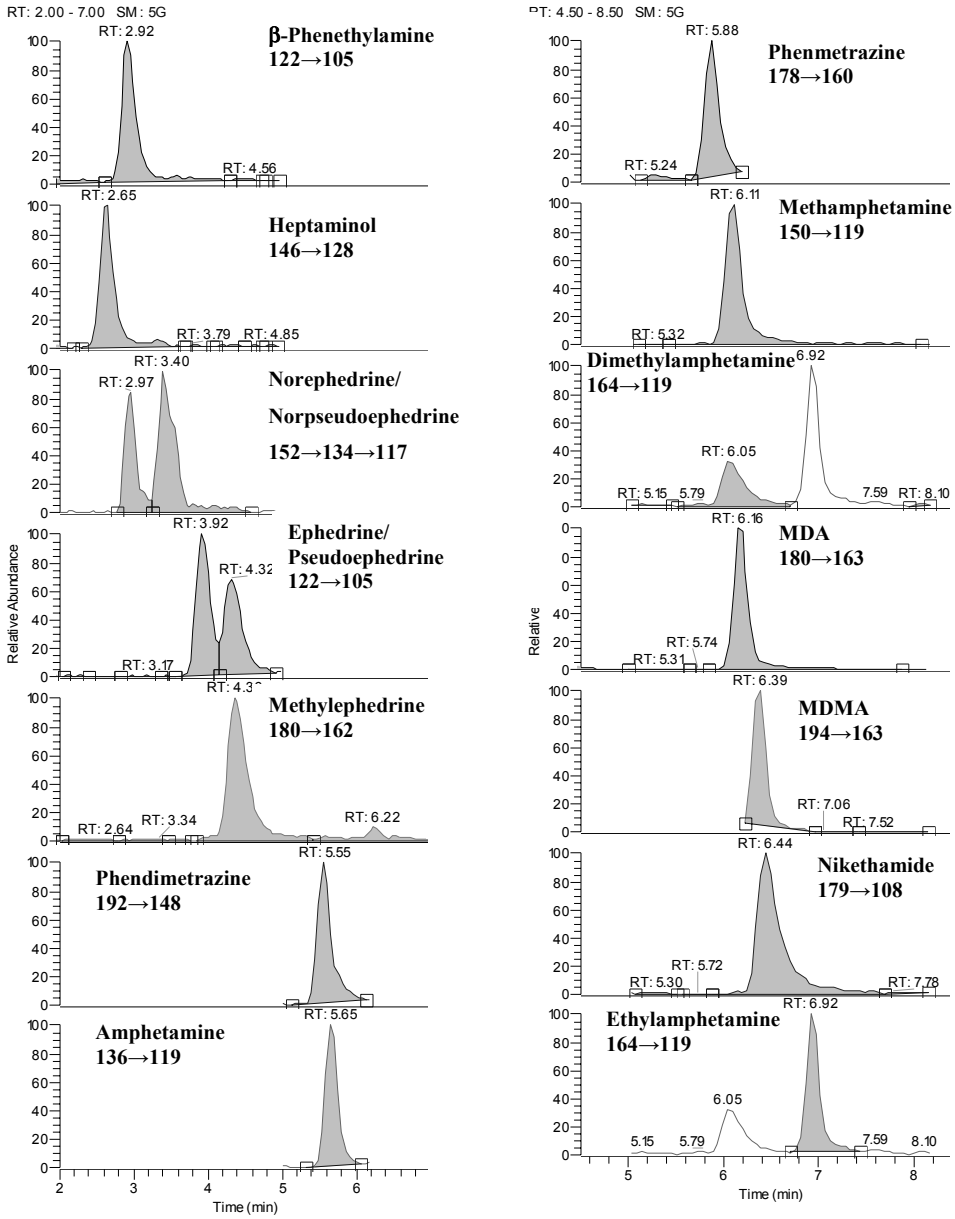
RT: 2.00 - 7.00 SM: 5G
β-Phenethylamine
122→105
Heptaminol
146→128
Norephedrine/
Norpseudoephedrine
152→134→117
Ephedrine/
Pseudoephedrine
122→105
Methylephedrine
180→162
Phendimetrazine
192→148
Amphetamine
136→119
Relative Abundance
Time (min)
RT: 4.50 - 8.50 SM: 5G
Phenmetrazine
178→160
Methamphetamine
150→119
Dimethylamphetamine
164→119
MDA
180→163
MDMA
194→163
Nikethamide
179→108
Ethylamphetamine
164→119
Relative
Time (min)

Figure 1(a)

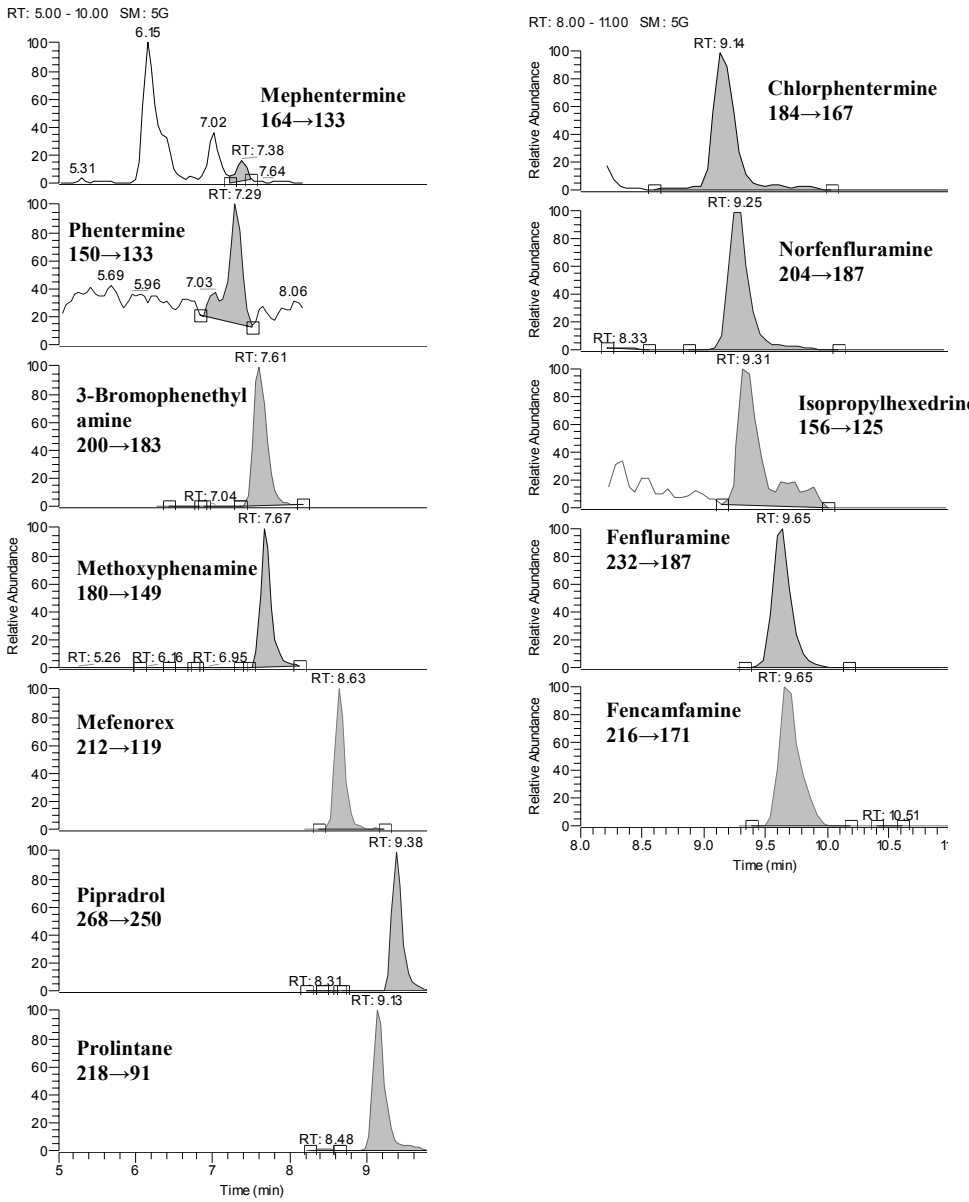

Figure 1(b)

Figure 1(a) and (b): Quality control urine spiked at 25 ng/ml (chlorphentermine at 250 ng/ml)

Conclusions

A sensitive LC-MS screening method for amphetamine and amphetamine type stimulants has been developed and validated. 7 MS^2 transitions were monitored simultaneously. Due to the sensitivity of this method the amount of urine routinely used for the extraction of stimulants could be reduced from 5 to 2 ml.
LC-MS seems to be a sensitive alternative for GC-NPD detection of amphetamines in doping analysis.

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3. Quantitative determination of strychnine in urine after ingestion of the homeopathic preparation *NUX Vomica TM*

Adapted from:

Van Eenoo P, Deventer K, Delbeke FT.
Quantitative LC-MS determination of strychnine in urine after ingestion of a Strychnos nux-vomica preparation and its consequence in doping control.
Forensic Sci. Int. 2006; **164**: 159.

Abstract

A simple, fast and sensitive method for the quantitative determination of strychnine in urine has been developed and validated. The method consists of a liquid-liquid extraction step with ethyl acetate at pH 9.2, followed by LC-MS^2 in positive APCI-mode. The method is linear in the range 1-100 ng/ml and allows for the determination of strychnine at sub-toxicological concentrations. The inaccuracy of the method ranged from 1.3 % to 4.4 %. The method was used to determine the excretion profile of strychnine after the ingestion of the over-the-counter herbal preparation *Strychnos nux-vomica*. Strychnine could be detected for 24 h. Maximum urinary concentrations ranged from 22.6 – 176 ng/ml. The results of this study show that the use of this type of preparations by athletes can lead to adverse analytical findings.

Introduction

The alkaloid strychnine was first isolated from *Strychnos ignatii* beans in 1818. The commercial source of strychnine however is the dried seed *Strychnos nux-vomica* [1]. Although strychnine is highly toxic [2], several homeopathic *Strychnos nux-vomica* preparations are available as over-the-counter products in Belgium.

Strychnine was one of the first substances used to enhance performance in sports. Already in the nineteenth century cyclists reportedly used cocktails of caffeine, cocaine, alcohol, ether and strychnine in endurance events [3] and the first reported drug-related death in sports (Arthur Linton in 1896) was probably due to strychnine [4]. Today, strychnine is still on the list of prohibited substance in sports [5]. An MRPL is defined for all stimulants (compound or main metabolite 0,5 µg/ml). Only for strychnine the MRPL is lowered to 0,2 µg/ml (specific exception) due to its effectiveness in low doses [6].

Several methods for the detection of strychnine in bio-fluids via gas chromatography [7-9] and liquid chromatography [10] have been published. These methods were mainly developed for toxicological purposes and the limits of detection were close to the MRPL of strychnine. Recently, methods using liquid chromatography-tandem mass

spectrometry (LC-MS^2) for the quantitative detection of strychnine in seeds [11] and insects [12] and the qualitative detection in urine [13] have been published. The limits of detection of these methods are lower than those previously developed for toxicological purposes. Taking into account the popularity and growth of the supplement/homeopathy market, it seems necessary to test if the implementation of this new technique would allow for the detection of strychnine after the use of over-the-counter preparations.

Experimental

Chemicals and reagents

Strychnine and nalorphine were purchased from Sigma (St.Louis, USA). Analytical grade potassium carbonate, sodium hydrogen carbonate and acetic acid were purchased from Merck (Darmstadt, Germany), acetonitrile was from Biosolve (Valkenswaard, The Netherlands), HPLC grade methanol from Acros (Geel, Belgium), ethyl acetate from Panreac (Barcelona, Spain) and HPLC grade water from Fischer (Loughborough, UK). The herbal preparation Nux-vomica MT was bought as an over-the-counter product in a local pharmacy and was from Homeoden-Heel (Drongen, Belgium).

Excretion study

The study was performed in four healthy male volunteers. The study protocol was reviewed and approved by the ethical committee of the institution (UZGent, Project EC/2005-81/sdp). Each volunteer signed a statement of informed consent. Each volunteer ingested 200 ml of water in which ten drops (equivalent to 380 µg of strychnine) of the over-the-counter Nux-vomica preparation were dissolved. Urine samples were collected before (0h) and quantitatively 2, 4, 6, 9 and 12 h after intake. Additional samples were taken 24, 36 and 48h after administration. All urine samples were either analyzed directly or stored at -20°C, awaiting analysis. Urinary pH, volume and density were measured and all samples were analyzed in duplicate. When

necessary, urine samples were diluted with water in order to obtain concentrations in the range of the calibration curve.

Sample preparation

The method was adapted from a previously published screening method for diuretics and beta-blockers [13]. The internal standard solution (50 µl nalorphine, 20 µg/ml) was added to 2.0 ml of urine and the urine was made alkaline with 200 mg $NaHCO_3$ /K_2CO_3 (2:1). Liquid-liquid extraction was performed by rolling for 10 min with 5 ml ethyl acetate. After centrifugation (1200*g*) the organic layer was transferred into a new tube and evaporated until dry under OFN at 40 ºC. The residue was dissolved in 200 µl mobile phase. Within each batch of samples, a blank urine sample, a system blank (aqua bidest) and a quality control sample (spiked at 10 ng/ml) were concurrently analysed.

Apparatus

Chromatography

Separation of the compounds was performed on a Microsorb cyanopropyl column, 100 mm x 4.6 mm, 3 µm equipped with a guard column 10 mm x 2 mm (both from Varian, Sint-Katelijne-Waver, Belgium), using a P4000 pump and a model AS3000 autosampler (TSP, San Jose,USA). The mobile phase consisted of acetonitrile and 1 % acetic acid in water. Gradient elution at a flow rate of 1.0 ml/min was as follows: 90% acetic acid (1%) decreased linear to 64% in 6.5 min followed by an increase to the initial acetic acid (1%) concentration, which was maintained for 5 min before the next injection (equilibration time). The total run time of the method was 12 min. The injection volume was 50 µl.

Mass spectrometry

Ionisation of the analytes was carried out on a LCQ-Deca instrument (Thermo, San Jose, USA) using atmospheric pressure chemical ionisation (APCI) in the positive

ionisation mode. The capillary temperature and evaporator temperature were maintained at 200 ºC and 300 ºC, respectively. The drying gas was maintained at 80 units while the auxiliary gas was set to 10 units. The capillary voltage was 10 V. The needle discharge current was arbitrarily set to 5µA.

For MS^2 experiments m/z=335 and m/z=312 were chosen as precursor ions in the APCI+-mode for strychnine and nalorphine, respectively. The relative collision energies for both compounds were 38 % and 36%, respectively. The isolation width for the precursor ion was set at 3. The activation q value and ionisation time were 0.250 and 30 ms, respectively.

Validation

A five-point calibration curve was generated by spiking blank urine with strychnine in triplicate at 1, 5, 10, 50 and 100 ng/ml. The area ratio of the product ions of strychnine (m/z = 264) and the internal standard (m/z = 270) were plotted versus the concentration. The precision and inaccuracy of the method were tested at three levels (1, 10 and 100 ng/ml). Precision was assessed as the percentage RSD of both repeatability (within-day) and reproducibility (between-day and different analysts) for a selected compound and level. Maximum allowed tolerances for reproducibility and repeatability can be calculated from the Horowitz-equation $RSD_{max} = 2^{(1-0.5\log C)}$ (C = concentration (µg/ml)*10^-6). The maximum allowed tolerances for repeatability and reproducibility are $2/3RSD_{max}$ and RSD_{max}, respectively [14].

Inaccuracy was defined as the difference between the calculated amount and the specified amount for the selected compound and expressed as a percentage [15].

Selectivity was tested by analysing reference mixtures containing several structurally related doping agents which are routinely screened for including alkaloids and stimulants. The concentration of these substances was 1 µg/ml. The LOQ of the method was defined as the lowest concentration where acceptable reproducibility and inaccuracy could be guaranteed. The LOD was defined arbitrarily as ½ LOQ. The product spectrum of strychnine in 6 samples spiked at the LOD was compared to a strychnine reference sample and conformity with the WADA guidelines [16] was checked (m/z 335, 307, 290 and 264 were used for comparative purposes).

For extraction recovery of strychnine, blank urine samples (n= 6) were spiked at 3 levels (1, 50, 100 ng/ml) and blank urine samples (n=6) were extracted. The extracts of the blank urine samples were spiked at the same levels simulating a 100% recovery. Finally, both sets of samples were evaporated and analysed with the described LC-MS method. The obtained peak areas of the two sets of samples were compared.

Results

The ion chromatograms and product spectra for strychnine and nalorphine in a quality control sample spiked at 10 ng/ml are shown in Figure 1.

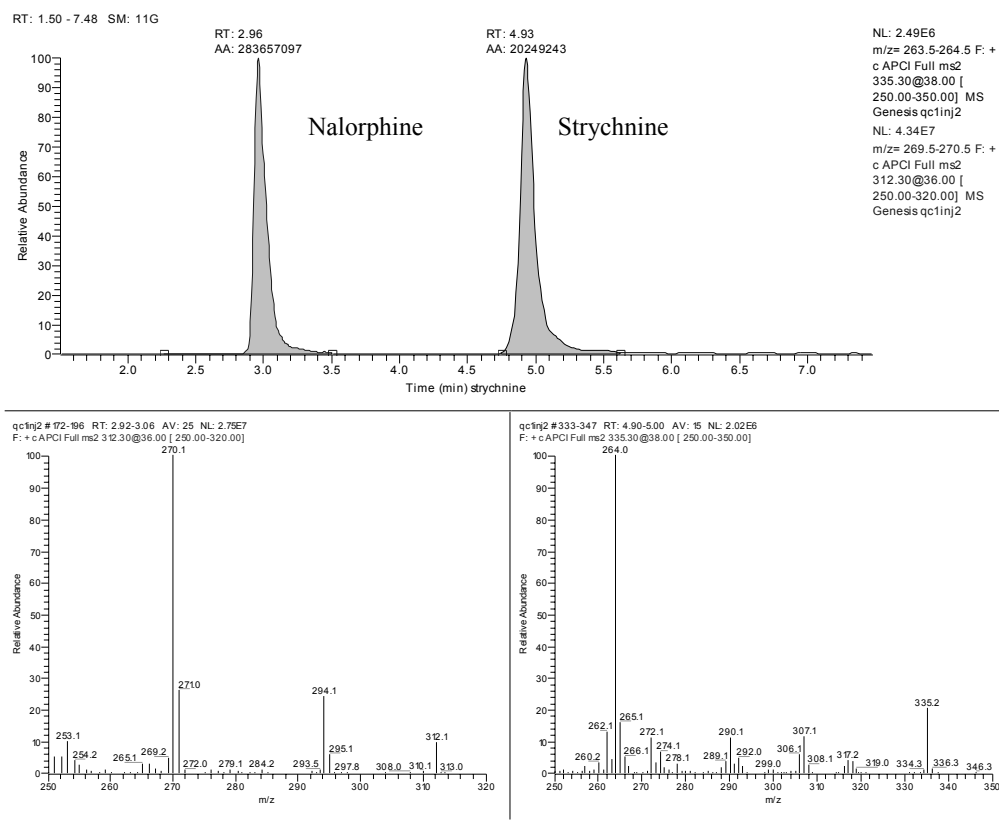

Figure 1: Ion chromatograms (m/z 270 and m/z 264) and product spectra of nalorphine (ISTD) and strychnine in a quality control urine spiked at 10 ng/ml.

The study was performed over a 2 months period and three calibration curves were generated. The correlation coefficients of the calibration curves always exceeded 0.99. Data on inaccuracy, repeatability and reproducibility are given in Table 1.

Table 1: Inaccuracy (between-day), repeatability, reproducibility and tolerance limits of the LC-MS method at three concentrations (1, 10 and 100 ng/ml).

Conc. [ng/ml]	Inaccuracy [%] *n=18*	Repeatability [%] *n=6*	Reproducibility [%] *n=18*	RSD_{max} [%]	2/3 RSD_{max} [%]
1	+ 1.3	2.5	11.5	45	30
10	+ 3.4	6.1	7.5	32	21
100	+ 4.4	3.9	6.4	23	15

The LOQ was 1 ng/ml, the LOD 0.5 ng/ml. The product spectra obtained in the samples spiked at the LOD were in agreement with the minimum requirements of WADA for the unequivocal identification of a prohibited substance. The extraction recoveries were 79.3 % ± 6.4 , 83.6 % ± 1.8 and 83.0 % ± 1.9 for 1, 10 and 100 ng/ml, respectively.
The excretion profiles in the 4 volunteers are shown in Figure 2 and the cumulative excretion profiles are given in Figure 3.

Discussion

The vast majority of reversed phase (in particular C18) HPLC separations take place on silica based stationary phases. However chromatography on silica results in poor peak shape for basic compounds. In particular for strychnine the problem of tailing peaks with silica based reversed phases is known [13]. Therefore a cyano-based column was applied instead of a C18-column. Since separation of polar compounds on this type of column is based on polar-polar interactions, tailing was less prevalent (Figure 1).
The fragmentation pattern in APCI (Figure 1) was comparable to results obtained with ESI in a previously published method for the detection of strychnine in seeds [11].
The concentration range of this method (1-100 ng/ml) was lower than the LOQs in other previously described methods. As shown in Table 1, the method allows for an accurate

and reproducible measurement of these low, sub-toxicological concentrations in urine in compliance with generally accepted criteria [14, 15]. The method is capable of quantifying strychnine in urine at levels expected in doping analysis. Maximum urinary strychnine concentrations (23-176 ng/ml) in the volunteers, would remain undetected via the methods routinely applied in toxicology [7, 9].

As shown in Figure 2 strychnine remained detectable for 24-48 h. The difference in maximum urinary concentration in volunteer 4 compared to the others (Figure 2) is probably caused by a difference in urine volume.

The cumulative excretion profiles (Figure 3) indicate that 2.2 to 8.6% of the ingested strychnine was excreted unchanged in urine during the first 12 h. These low amounts excreted are in agreement with previously published results [17].

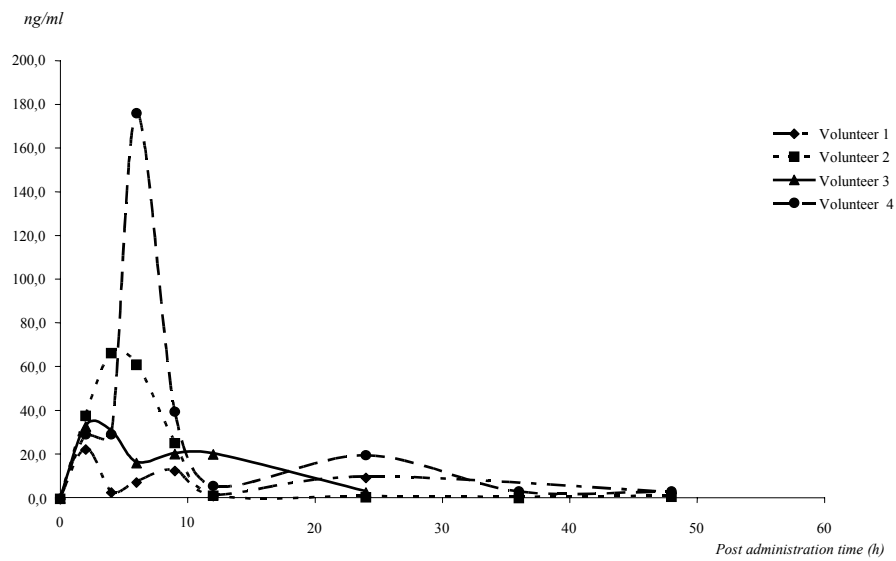

Figure 2: Urinary excretion profile of strychnine in the urine samples from 4 volunteers (380 µg of strychnine administered as a *Strychnos nux vomica* extract)

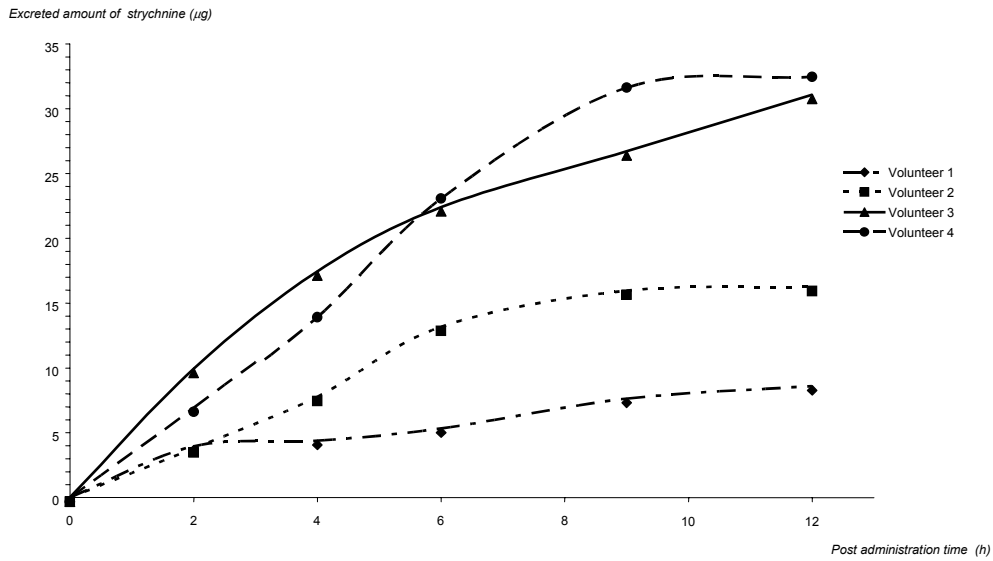

Figure 3: Cumulative excretion of strychnine in urine within the first 12 h post administration of 380 µg in 4 volunteers.

Conclusions

A LC-MS method for the quantitative determination of strychnine in urine after the ingestion of sub-toxicological concentrations of an over-the-counter *Strychnos nux-vomica* preparation was developed. The method allowed for the detection of strychnine up to 48 h after the administration of 380 µg. The use of over-the-counter preparations containing *Strychnos nux-vomica* extracts could lead to adverse analytical findings in doping analysis.

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General Discussion

LC-MS, an evolution in doping analysis

Before the introduction of LC-MS in the field of doping analysis, mass spectrometrical information was acquired with capillary GC-MS. This analytical technique is only suitable for volatile compounds. Because doping agents include polar functional groups in their structure, the volatility is limited and prior to GC-MS analysis, derivatisation is required for most of them. Because GC-MS has been the standard technique in doping analysis for more than 30 years the derivatisation techniques were well optimised for anabolic steroids, narcotics and beta-blockers [1,2]. Unfortunately, some derivatives are thermal labile and can not be detected.

Diuretics include polar compounds with a wide variety of physico-chemical properties and can not be derivatised with a single derivatisation agent. Therefore, they were analysed by HPLC-UV. Because LC-MS allows to detect polar compounds without derivatisation, the first doping related LC-MS application was described for this group [3]. Despite the sensitivity of this application and the straightforward sample preparation, two consecutive runs in both polarities were necessary to detect all species from this group. By the end of the 1990s LC-MS instruments allowing reliable and fast polarity switching became available and positively as well as negatively charged diuretics could be detected in a single chromatographic run.

The routine detection method for the diuretics by means of LC-MS came available in our laboratory by the end of 2001 [4]. Initially, this screening method contained 18 diuretics and probenecid. Currently, this method contains 36 diuretics and 21 beta-blockers.

Other, non diuretic compounds which require an acidic extraction were also included in this screening method, i.e. modafinil-acid (metabolite of adrafinil and modafinil, narcoleptic agents), ritalinic acid (metabolite of methylphenidate, used in the treatment of ADHD) and carboxy-finasteride (metabolite of finasteride, an aromatase inhibitor).

The thermal lability and low volatility of corticosteroids make them only amenable for GC-MS analysis after derivatisation. Moreover, due to the presence of multiple hydroxyl functions, a long derivatisation time is required.
Therefore corticosteroids represent another class of doping agents where the potential of LC-MS was fully exploited. Research revealed that with LC-MS a sensitivity could be reached impossible with any other analytical technique [5].
Soon after the introduction of the routine screening method for corticosteroids in our laboratory in the summer of 2002, corticosteroids were frequently detected (Table 1).

Table 1: Number of detected corticosteroids at DoCoLab (1999-2005)

Year	1999	2000	2001	2002	2003	2004	2005
Number	0	4	1	12	43	46	41

The sensitivity of the method allowed us not only to detect systemic abuse of corticosteroids but also residual amounts of corticosteroids originating from long lasting intra-articular applied depot preparations as well as corticosteroids from dermatological applications. Systemic use of corticosteroids is prohibited, topical applications are allowed. Other routes of administration (intra-articular, inhalation,...) are also allowed, but require an abbreviated TUE [6,7]. To prevent reporting low corticosteroid concentrations, related to topical application, WADA introduced in 2005 a scientifically unsound reporting level of 30 ng/ml. Concentration above this limit are considered as resulting from non topical use. Unfortunately there is no scientifical evidence available for the concentration of 30 ng/ml. As an answer to this reporting level different laboratories have initiated research concerning the detection of corticosteroids following several administration routes. In our laboratory, the detection of budesonide after inhalation was investigated. The results presented in this work show that not all volunteers in the experiment exceed the reporting level after the administration of a commercial preparation for which an abbreviated TUE is required.
Moreover, research at the Italian doping laboratory revealed that after oral intake (prohibited) the reporting level is only reached for a short period [8].

The breakthrough of LC-MS in the screening of anabolic steroids in our laboratory was achieved with the detection of tetrahydrogestrinone (THG), a designer anabolic steroid. This compound could not be detected using traditional GC-MS screening method again due to derivatisation problems.
Hence by the end of 2003 our doping laboratory had developed a LC-MS^2 screening method for this compound. This method includes currently also the detection of 3'hydroxystanozolol. This compound is an important metabolite for the detection of stanozolol, a potent and frequently used anabolic agent.

Stimulants are traditionally screened for underivatised by GC-NPD. Unfortunately, sensitivity and selectivity are limited with this technique. Therefore the stimulants were transfered to GC-MS detection and were included into the screening method for narcotics.
This method showed sufficiant sensitivity and sensitivity and LC-MS detection of this group of compounds was not required. Nevertheless, many methods devoted to the analysis of one or more stimulants are available in clinical and forensic toxicology.
In 2004 DoCoLab got involved in a project for the analysis of nutritional supplements [9]. In this project stimulants had to be analysed by LC-MS. Therefore a screening method was developed [10]. Because this method was readily available and no screening method for stimulants for anti-doping purposes was described so far we decided to apply this method to the urinary detection of stimulants. The application to urine showed that LC-MS can also be used as a sensitive alternative for the GC-MS method currently available in the laboratory.

Reversed-phase liquid chromatography is the most common applied technology for the separation of all kind of compounds and was consequently used in this work except for the detection of strychnine where a cyanopropyl-column was used in order to obtain improved peak shape. One of the major advantages of mass spectrometry is its inherent specificity and selectivity. As a consequence chromatographic separation can be reduced to the required minimum and shorter columns can be applied, resulting in higher sample throughput and shorter chromatographic run times. Hence the screening methods presented in this work, using 100 mm columns, are currently being adapted to

these 50 mm columns allowing faster analysis. In the last year conventional column dimensions and particle sizes seem to be a thing from the past and a new trend in chromatography was introduced, i.e. ultra fast separations using higher flow rates, higher column temperature and small column particles (1,8 µm).

Throughout this work two LC-MS interfacing techniques were applied, namely ESI and APCI. Typically, ESI is the preferred interface for polar compounds and APCI for non-polar compounds [11]. However, this theoretical assumption is not always valid. Corticosteroids and stanozolol, non-polar steroids, exhibited better sensitivity with ESI whereas for glucose, a highly polar compound, better sensitivity is observed with APCI [12]. Therefore both interfaces should be tested for all investigated compounds.
Nevertheless, the choice for an interface throughout this work was always determined by the compound with the poorest detection. Where no difference was observed between ESI and APCI, the latter technique was prefered regarding its robustness to matrix suppression [13].
Matrix suppression is caused by coelution of the biological matrix or other compounds with the analyte of interest, and it may be problematic especially in high-throughput LC-MS analysis where the analyte is only poorly separated from unretained matrix. This phenomena was observed during development of a screening method for THG.
Despite the multiple interfacing techniques presented in the past [14], ESI and APCI are the only two routinely used nowadays. However new interfacing techniques have been suggested including APPI and APLI. The former ionisation technique has proven to be unsensitive for anti-doping purposes [15] whereas the latter has not yet been evaluated.

In this work all experiments were performed using ion trap technology. Ion trap technology exhibits high sensitivity when operated in full scan mode and allows for multiple stages mass spectrometry (MS^n). Full scan MS was applied in the screening method for diuretics and beta-blockers and the quantitation of 6-OXO-AD. MS^2 was applied for the screening of corticosteroids, anabolic steroids and stimulants. Nevertheless, for unambiguous confirmation of a suspected sample at least three diagnostic ions should be present in the mass spectrum fulfilling WADA- regulations [16]. Therefore confirmation is exclusively performed using full scan MS^2 or MS^3.

Although the popularity of ion trap mass spectrometry is mostly concentrated on qualitative analyses, these mass spectrometers perform well for quantitative analyses as can be concluded from the results regarding the detection of 6-oxo-AD, strychnine and budesonide.

Future developments

In this work, different LC-MS methods for the detection of doping substances in urine have been developed and further optimised. The screening methods for diuretics, beta-blockers, corticosteroids and selected anabolic steroids were successfully implemented in our routine monitoring program. At this moment GC-MS is still the standard screening method for narcotics, stimulants and the majority of anabolic steroids. Nevertheless, the routine applicability of LC-MS for the stimulants has been suggested in this work and the comprehensive screening for narcotics and anabolic steroids is in preparation. Hence, it is foreseeable that the application of LC-MS in anti-doping analysis will continuously increase.

Because LC-MS allows to detect polar non-volatile compounds, this technique has a great potential in the analysis of peptide hormones. Currently, work concerning their detection is being initiated in our laboratory.

Since the expanded use of LC-MS, the number of manufacturers producing LC-MS systems has increased and consequently the purchase costs are decreasing.
Hence LC-MS techniques, formerly budgetary out of reach, are becoming affordable. One of these techniques which is extremely powerful is TOF-MS, where a mass spectrometer is combined with a time-of-flight (TOF) instrument. This technology is very promising in structure elucidation of unknown compounds due to the accurate mass measurement. Recently developed TOF analyzers also provide an interesting tool for peptide detection due to their extended mass range. Using accurate mass measurement together with classical MS^n technology can reveal the chemical structure of unknown new doping substances.

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Summary

In the first part of **Chapter I** the history of doping is described. The first doping offences were found in the Roman Circus Maximus where the ancient Roman gladiators used stimulants mixed with alcohol. Organised doping abuse was first reported during the second part of the 19th century and amphetamines were popularised after the Second World War. In the second half of the 20th century anabolic steroids, diuretics and narcotics found their way to the athletes. Nowadays doping laboratories are confronted with more sofisticated substances including peptide-hormones and designer steroids.
Liquid chromatography–mass spectrometry, the analytical technology used to realise this work is highlighted in the second part of the first chapter. Although the principles of LC and MS were already known at the beginning of the 20th century, the coupling of these two techniques was achieved by the end of the 1960s. Affordable and reliable commercial instruments were available in 1990s. Since this work includes several validated screening methods, the principles and criteria for validation related to doping analysis were also explained in this chapter.

In the first part of **Chapter II** a comprehensive and selective LC-MS$^{(2)}$ method for the screening of 18 diuretics and probenecid in human urine is presented. Analysis was performed using scan by scan polarity changing. All diuretics and probenecid were separated in less than 20 minutes after liquid/liquid extraction with ethyl acetate. The LOD for all substances was at least 100 ng/ml. The method was applied to the detection of diuretics after the oral administration of several commercial preparations. All diuretics were detectable for at least nine hours after intake.
In the second part of this chapter the screening method for the diuretics is extended with 8 other diuretics and 21 beta-blockers allowing simultaneous determination of two important groups of anti-hypertensive drugs in human urine.

Chapter III is dedicated to corticosteroids. In the first part of this chapter a selective and sensitive method for the screening of 9 corticosteroids in human urine is presented.

All corticosteroids were separated in less than 20 min after liquid/liquid extraction with diethylether. The limit of detection for all substances was 4 ng/ml or lower.
In a second part of this chapter the validated method was applied for the detection of several corticosteroids after administration. This study shows that corticosteroids can be detected in urine following oral, nasal, bronchial and parenteral applications.
In the last part of this chapter the detection of budesonide after inhalation was described. For this purpose a sensitive and accurate LC-MS^2 method was developed and validated. After administration of 200 µg of budesonide to 5 healthy volunteers budesonide could not be detected in any urine sample whereas 16α–hydroxyprednisolone (its major metabolite) was detectable up to 12 hours post administration.

The detection of anabolic steroids is investigated in **Chapter VI**. Sample preparation consists of a liquid/liquid extraction with diethylether after enzymatic hydrolysis. Analysis was performed using electrospray ionisation. MS^2 was applied for all compounds. The analytical run time was 11 minutes. The LOD for all compounds varied between 1 and 10 ng/ml.
In a second part of this chapter the detection of 6-OXO®, a nutritional supplement commercially available on the internet, is investigated. 6-OXO® contains androst-4-ene-3,617-trione which can act as an aromatase-inhibitor. A sensitive LC-MS method was developed for its detection. Using this method androst-4-ene-6α-ol-3,17-dione was identified as the major urinary metabolite, and androst-4-ene-6α,17β-diol-3-one as a minor metabolite.

Chapter V deals with the detection of stimulants. The first part of this chapter describes a selective and sensitive method for the qualitative screening of urine samples for 27 amphetamine and amphetamine-type drugs in the field of doping analysis. The method consists of a liquid/liquid extraction with diethylether at pH 14 using APCI in positive ionisation mode. The total run time was 15 minutes. All compounds were analysed in MS^2 or MS^3. The detection limit for all compounds was lower than 25 ng/ml except for chlorphentermine (LOD: 250 ng/ml).

A second part of this chapter presents the detection of strychnine after the intake of the homeopathic preparation *Strychnos nux-vomica*. The method consists of a liquid/liquid extraction step with ethyl acetate at pH 9.2, followed by LC-MS^2 in positive APCI-mode. Strychnine could be detected for 24 h. Maximum urinary concentrations ranged from 22.6 – 176 ng/ml. The results of this study show that the use of this type of preparations by athletes can lead to adverse analytical findings.

The results as well as the experiences with LC-MS during the 5 years of research are described in the **General Discussion**. This work documents that LC-MS can be succesfully applied for the sensitive detection of diuretics, beta-blockers, corticosteroids, selected anabolic steroids and stimulants. Furthermore, due to its ability to detect non-volatile high molecular weight compounds, LC-MS is a promising tool in the detection of peptide hormones and nucleotide based doping agents as well.

Samenvatting

In het eerste deel van **Hoofdstuk I** wordt de geschiedenis van doping besproken. De eerste meldingen van dopinggebruik zijn te vinden bij de Romeinse gladiatoren. Zij gebruikten een mengsel van alcohol en stimulantia om zich op te peppen. Amfetamines werden na de Tweede Wereldoorlog op grote schaal misbruikt in de sport. Eén van de dieptepunten van amfetaminemisbruik was de dood van wielrenner Tom Simpson. Hij overleed in 1967 op de flank van de Mont Ventoux. Tijdens de jaren '60 en '70 vonden ook anabolica, narcotica, diuretica en beta-blockers hun weg naar de sport.
Het tweede deel van dit hoofdstuk geeft een theoretische beschrijving van de technologie die aan de basis van dit werk ligt, namelijk vloeistofchromatografie-massaspectrometrie (LC-MS). De principes van vloeistofchromatografie en massaspectrometrie waren reeds gekend aan het begin van de 20^{e} eeuw maar de koppeling van de twee technieken liet op zich wachten tot het einde van de jaren zestig. Betaalbare, commerciële toestellen kwamen beschikbaar tegen het midden van de jaren negentig.
Aangezien verschillende hoofdstukken van dit werk de ontwikkeling van gevalideerde methodes behandelen, worden de relevante criteria voor kwalitatieve methodevalidatie in het kader van dopinganalyse besproken in het derde deel van dit hoofdstuk. De belangrijkste parameters zijn: MRPL, LOD, selectiviteit en specificiteit.

In het eerste deel van **Hoofdstuk II** wordt een methode beschreven voor het opsporen van diuretica in humane urine met behulp van LC/MS$^{(n)}$. De toepassing van scan-to-scan polariteitsomwisseling laat toe om alle diuretica te detecteren in één analyse. De detectielimieten zijn voor alle componenten lager dan 100 ng/ml. Vervolgens werd de methode gebruikt om excretie-urines te analyseren. De resultaten van de excretieproeven tonen aan dat diuretica tot ten minste negen uur na toediening opgespoord kunnen worden.
Het tweede deel van dit hoofdstuk beschrijft de uitbreiding van de screeningsmethode met 21 beta-blockers. De uitbreiding resulteerde in een screeningsmethode waarmee

twee groepen van bloedrukverlangende middelen in één analyse kunnen opgespoord worden.

In **Hoofdstuk III** worden de corticosteroïden behandeld. De ontwikkeling en validatie van een LC-MS^2 methode voor de detectie van 9 corticosteroiden wordt in het eerste deel van dit hoofdstuk beschreven. Als staalopzuivering werd een vloeistof/vloeistof extractie gebruikt met diethylether. Voor alle corticosteroiden werden detectielimieten lager dan 4 ng/ml bekomen en de analysetijd van de methode bedraagt 20 minuten. Door het grote aantal corticosteroïden die gedetecteerd werden met de screeningsmethode besloten we om in een tweede deel van dit hoofdstuk de detectietijden van corticosteroiden na orale, nasale, bronchiale en parenterale toediening te onderzoeken. Uit de resultaten bleek dat alle toedieningsroutes konden leiden tot een positieve dopingtest. De langste detectietijden werden bekomen voor de geïnjecteerde corticosteroïden. Deze konden teruggevonden worden tot 20 dagen na toediening.
In het laatste deel van dit hoofdstuk wordt de urinaire detectie van budesonide en metaboliet 16α–hydroxyprednisolone na inhalatie onderzocht. Vijf gezonde vrijwilligers inhaleerden elk een éénmalige dosis van 200 µg budesonide. In geen enkel urinestaal kon budesonide gedetecteerd worden. De hoofdmetaboliet 16α–hydroxyprednisolone kon gedetecteerd worden tot 12 uur na toediening.

De detectie van anaboliserende stoffen wordt behandeld in **hoofdstuk VI**.
In het eerste deel van dit hoofdstuk wordt een LC-MS methode-ontwikkeling voor het routinematig opsporen van anabolica in urine besproken. De analyses werden uitgevoerd met behulp van electrospray ionisatie. Om maximale gevoeligheid te behalen, werden alle analyses uitgevoerd in MS^2. De totale analysetijd bedraagt 11 minuten en de LOD voor alle componenten varieert tussen 1 en 10 ng/ml.
6-OXO® is een nieuw voedingssuplement dat te koop wordt aangeboden via het internet. Het wordt verkocht als aromatase inhibitor en bevat androst-4-ene-3,6,17-trione als actief ingrediënt. In het tweede deel van dit hoofdstuk wordt de detectie van deze component met behulp van LC-MS besproken. De ontwikkelde LC-MS methode werd succesvol toegpast voor het identificeren van twee metabolieten, namelijk androst-

4-ene-6α-ol-3,17-dione (hoofdmetaboliet) en androst-4-ene-6α,17β–diol-3-one (slechts in lage concentraties terug te vinden).

In het eerste deel van **Hoofdstuk V** wordt een LC-MS^n methode besproken voor het opsporen van 27 amfetamines en amfetamine-analogen in humane urine.
De totale analysetijd bedraagt 15 minuten en de detectielimieten varieerden tussen de 1 en 25 ng/mL met uitzondering van chloorfentermine (detectielimiet 250 ng/mL).
In het tweede deel van dit hoofdstuk wordt een éénvoudige analytische methode beschreven om strychnine in urine op te sporen. De methode bestaat uit een vloeistof/vloeistof extractie met ethylacetaat gevolgd door LC-MS^2 analyse. Vervolgens werd een excretieprofiel voor strychnine opgesteld na de inname van het homeopatisch product *Strychnos nux-vomica*. Strychnine werd tot 48 uur na inname gedetecteerd. De maximale concentraties varieerden tussen 22,6 en 176 ng/mL. Deze studie toont aan dat homeopatische preparaten tot positieve dopingresultaten kan leiden.

Curriculum Vitae

Koen Renaat Gilbert Deventer werd geboren op 28 juni 1976 in Ronse. Na het behalen van het diploma hoger secundair onderwijs aan het Onze Lieve Vrouwecollege te Oudenaarde (Wetenschappelijke B) begon hij in 1994 met de studies van industrieel ingenieur aan de Katholieke Hogeschool Gent en behaalde het diploma industrieel ingenieur scheikunde in 1998 met voldoening. Na 1,5 jaar industriele ervaring bij Omnichem (Wetteren) en Coca-Cola Beverages (Zwijnaarde) begon hij in 1999 met de studie licentiaat scheikunde aan de universiteit Gent en behaalde het diploma licentiaat scheikunde in 2001 met onderscheiding. Onmiddellijk daarna trad hij in dienst als doctoraatsbursaal bij het dopingcontrolelaboratorium van de universiteit Gent.

Zijn taak binnen het laboratorium bestond erin het LC-MS departement uit te bouwen in het kader van dopingonderzoek.

Initieel was het de bedoeling zich toe te leggen op de detectie van corticosteroïden. Al snel werden andere groepen dopeermiddelen toegevoegd aan het onderzoek.

Het eigenlijke onderzoek werd gestart in 2001 en heeft uiteindelijk tot dit proefschrift geleid. Hij is auteur of mede-auteur van 12 publicaties in internationale en nationale tijdschriften en nam actief deel aan verschillende internationale congressen.

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Poster Presentation:

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